Investigation of Local South African Avipoxviruses as Potential Vaccine Vectors

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Declaration

The study described in this thesis was performed in the Division of Medical Virology, Department of Clinical Laboratory Sciences of the University of Cape Town, under the supervision and guidance of Professor Anna-Lise Williamson and Dr Nicola Douglass. This is my own work and the use of information from others has been referenced. The assistance received from others has also been acknowledged.

Signed by candidate

Kristy Offerman

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Abstract

Avipoxviruses are large, genetically diverse DNA viruses which are particularly desirable for use as vaccine vectors as a result of their excellent safety profile and host range restriction.

In this study, 8 novel South African (SA) avipoxvirus isolates were characterized. They could be divided into five groups, according to gross pathology and pock appearance on CAMs. Histopathology revealed distinct differences in epidermal and mesodermal cell proliferation, as well as immune cell infiltration, caused by the different avipoxviruses. Phylogenetic analysis was performed based on several conserved poxvirus genetic regions, corresponding to vaccinia virus (VACV) A3L (fpv167 locus, VACV P4b), G8R (fpv126 locus, VLTF-1), H3L (fpv140 locus, VACV H3L) and A11R–A12L (fpv175–176 locus). The SA isolates all grouped in clade A, either in subclade A2 or A3 of the genus Avipoxvirus, with branching patterns which differed according to the locus analysed.

An in-depth characterisation of a novel SA avipoxvirus from a Feral Pigeon (*Columba livia*) (FeP2) was performed. The growth kinetics and morphogenesis of FeP2, as well as the ability of this virus to induce/inhibit apoptosis was investigated in permissive and non-permissive cell lines. FeP2 was shown to grow productively in primary CEF cells and exhibit complete morphogenesis in these cells. Conversely, growth of FeP2 was inhibited in mammalian cells and the block in morphogenesis in human cervix adenocarcinoma (HeLa) cells occurred prior to the formation of immature virus. FeP2 was shown to inhibit apoptosis in both permissive CEFs and non-permissive HeLa cell lines.

The full genome of FeP2 was sequenced and compared to the published canarypox virus (CNPV) and fowlpox virus (FWPV) sequences. Phylogenetic as well as in-depth sequence analysis supports the hypothesis that FeP2 evolved independently from an ancestral virus common to FWPV and CNPV. The FeP2 sequence (282kbp) shares 85.3% nucleotide identity with FWPV and 62.0% nucleotide identity with CNPV. Overall, FeP2 and FWPV have syntenic gene arrangements; however, major differences exist throughout their genomes, the most striking of which is a large deletion of ~16kbp from the central region of the genome of FeP2 deleting a cc-chemokine-like gene, two variola virus B22R orthologues, an N1R/p28-like gene and a V-type Ig domain family gene, all of which are involved in host range and/or

immunomodulation. FeP2 encodes orthologues of vaccinia virus C7L and Interleukin 10.

In order to investigate the effect of different poxviruses on mammalian host gene expression, we compared host gene expression profiles in the spleens of BALB/c mice in response to infection (10^5 pfu/mouse) with six different poxviruses, namely Lumpy Skin Disease virus (LSDV), wild type CNPV, FWPV (DCEP 25 modified strain), modified vaccinia Ankara (MVA), PEPV and FeP2. The findings presented here indicate that six, genetically diverse host-restricted poxviruses produce qualitatively and quantitatively distinct host responses in an *in vivo* mouse model. Differential gene expression associated with the different poxviruses is discussed with emphasis on immunity-related responses. Of particular interest, CNPV and FWPV induce the up regulation of two immunoglobulin genes (Ighg and Ighg3 (IgG3)) with CNPV inducing a third, Ighm (IgM). HIV-1–specific IgG3 antibodies have been found to correlate with decreased risk of HIV-1 infection in the RV144 trial, which included a CNPV-based vector. Up regulation of IgG3 by CNPV and FWPV but not the other poxviruses tested *in vivo*, implies that these two avipoxvirus-vector backbones may be involved in stimulation of the clinically important IgG3 antibody subclass.

The work presented here provides a basis from which to expand and further investigate the role of novel poxviruses in the development of additional vaccine vectors.

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List of abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
AIDS	acquired immunodefiency syndrome
ALVAC	canarypox virus vaccine strain
APC	antigen-presenting cells
APV	Avipoxvirus
ATCC	American Type Culture Collection
BCL-2	B cell lymphoma 2
BHK-21	Syrian baby hamster kidney cells
bp	base pairs
BPSV	bovine papular stomatitis virus
CAM	chorioallantoic membrane
Casp 1	caspase 1
CEFs	chick embryo fibroblasts
CEV	cell associated enveloped virus
ChPV	*Chordopoxvirinae*
CNPV	canarypox virus
CPE	cytopathic effect
CrmA	cytokine response modifier A
CT	Cape Town
CTLs	cytotoxic T lymphocytes
CV-1	simian kidney cells
CVA	chorioallantoid vaccinia Ankara
CPV	cowpox virus
DAMPs	danger-associated molecular patterns
DAVID	Database for Annotation, Visualization and Integrated Discovery
DC SIGN	DC-specific ICAM-3–grabbing nonintegrin
DCs	dendritic cells
DISC	death-inducing signalling complex
DMEM	Dulbecco`s modified Eagle‘s medium
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
dVV-L	defective vaccinia Lister
ECTV	ectromelia virus
EEV	extracellular enveloped virion
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbant assay
EnPV	*Entomopoxvirinae*
FADD	FAS-associated death domain protein
FC	fold change
FCS	fetal calf serum
FeP1	Feral Pigeon isolate 1
FeP2	Feral Pigeon isolate 2
FP9	attenuated European strain of fowlpox virus derived from European FWPV HP1
FPVUS	pathogenic American strain of fowlpox virus
FWPV	fowlpox virus
GO	gene ontology
GPCRs	G protein–coupled receptors
HeLa	human cervix adenocarcinoma cells
HIV-1	human immunodeficiency virus subtype 1
HP1	pathogenic European strain of fowlpox virus
i.v.	intravenously
ICAM-3	intracellular adhesion molecule-3
ICTV	International Committee on Taxonomy of Viruses
IEV	intracellular enveloped virus
Ifi27l2a	interferon, alpha-inducible protein 27 like 2A
IFN	interferon
Igh	immunoglobulin heavy chain gene
IgK	immunoglobulin kappa chain complex

IL-10	interleukin-10
IL-1β	interleukin-1 beta
IL-18	interleukin-18
IL-18BP	IL-18 binding proteins
IRF-1	interferon regulatory factor 1
ISGA	Integrated Services for Genomic Analysis
ISGs	interferon stimulated genes
ITRs	inverted terminal repeats
IV	immature virus
LAIV	live attenuated influenza vaccine
LD1	laughing dove isolate 1
LD2	laughing dove isolate 2
LSDV	lumpy skin disease virus
Ly96	lymphocyte antigen 96
m.o.i.	multiplicity of infection
Marco	macrophage receptor with collagenous structure
MCP1	monocyte chemotactic protein 1
MCV	molluscum contagiosum virus
MDA5	melanoma differentiation associated gene 5
MDBK	Madin Darby bovine kidney cells
MDDCs	monocyte derived dendritic cells
MDV	Marek‘s disease virus
MHC	major histocompatibility complex
ML	maximum likelihood
Mov-10	moloney leukemia virus 10
mRNA	messenger RNA
MV	mature virions
MVA	modified vaccinia Ankara
MXPV	monkeypox virus
MYXV	myxoma virus
NLRs	nucleotide-binding and oligomerization domain (Nod)-like receptors
nt	nucleotide
NYVAC	vaccinia virus vaccine strain with specific deletions
NZ	New Zealand
OPV	Orthopoxvirus
ORFs	open reading frames
PAMPs	pathogen associated molecular patterns
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBS + PSF	phosphate buffered saline + penicillin, streptomycin and fungin
PCR	polymerase chain reaction
PE	Port Elizabeth
PEPV	penguinpox virus
pfu	pock forming units
PGPV	pigeonpox viruses
Pi5	Racing Pigeon isolate 5
PKR	dsRNA-activated protein kinase
PRRs	pattern-recognition receptors
QM7	Japanese quail myoblast cell line,
REV	reticuloendotheliosis virus
RIG-I	retinoic acid-inducible gene-I
RLRs	retinoic acid-inducible gene-I (RIG-I)-like receptors
RMA	robust multi-array averaging
RNA	ribonucleic acid
ROS	reactive oxygen species
RP1	Rock Pigeon isolate 1
RP2	Rock Pigeon isolate 2
RT-PCR	real time polymerase chain reaction
SA	South Africa
SP1	juvenile Rock Pigeon (Speckled) isolate 1

SPF	specific pathogen–free
TANV	tanapox virus
TEM	transmission electron microscope
TGF-β1	transforming growth factor β1
TIV	trivalent influenza vaccine
TLRs	toll-like receptors
TMPK	thymidylate kinase
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
UCT	University of Cape Town
VACV	vaccinia virus
VACV-COP	VACV, Copenhagen strain
VACV-WR	vaccinia virus, Western Reserve strain
VARV	variola virus
vCP205	ALVAC-HIV recombinant containing clade B GagProt and gp120MN
VEGF	vascular endothelial growth factor
VGF	VACV growth factor
WHO	Assembly of the World Health Organization
WV	wrapped virus
YLDV	yaba-like disease virus
α-SNAP	α-type soluble NSF attachment proteins
β-NGF	β-nerve growth factor

Chapter 1

Literature review

1.1 Poxviruses

1.1.1 Classification

The *Poxviridae* are a family of complex DNA viruses which replicate solely in the cytoplasm of host cells [1]. The *Poxviridae* family consists of two subfamilies; the *Chordopoxvirinae* (ChPV) which are specific to vertebrate hosts, and the *Entomopoxvirinae* (EnPV) which infect insects. The ChPV subfamily can be further divided into nine genera (Table 1.1) and the EnPV subfamily into three genera (Alphaentomopoxvirus, Betaentomopoxvirus and Gammaentomopoxvirus) [2].

The evolutionary relationships between members of the *Poxviridae* family have been based on phylogenetic analysis and genome content [3, 4]. The EnPVs are divergent amongst themselves and compared to the ChPVs [3]. Within the ChPV subfamily, the Avipoxviruses, Parapoxviruses, molluscum contagiosum virus (MCV) and crocodilepox virus (CRV) are considerably divergent from the others [5]. According to analysis of the conserved DNA polymerase amino acid sequences, the Avipoxviruses and Parapoxviruses share the lowest percentage identity (40-50%). CRV also has <50% aa identity to other chordopoxviruses and is most closely related to MCV [5]. Orthopoxviruses, Yatapoxviruses, Cervidpoxvirus (Deerpox virus), Suipoxviruses and Capripoxviruses share the highest intergenus percentage amino acid identity (65-80%). Within both the Orthopoxvirus and Capripoxvirus genera, virus species share between 97-99% identity. Species within the Parapoxviruses and Yatapoxviruses share approximately 85% identity whereas members of the Avipoxvirus genus (canarypox and fowlpox viruses) share 79% amino acid identity [8]. For the purpose of this review, focus has been placed on two specific genera within the ChPV subfamily; the Orthopoxviruses (OPV) and the Avipoxviruses (APV).

1.1.2 Poxvirus genome and morphology

Poxviruses are large, ~~b~~rickshaped" viruses ranging between 200-400nm in length and which contain a dumbbell-shaped, internal core consisting of the genome and lateral bodies of heterogeneous material between the core and the outer membrane [1].

Poxviruses have linear, double-stranded DNA genomes between 130 and 300kbp in size [1]. Inverted terminal repeats (ITRs) of variable size consisting of homologous

sequences exist in opposite orientations on each end of the genome and terminal hairpin loops are present which connect the two DNA strands [1, 6]. Genes within these ITRs are present as diploid copies. Within the OPV, the size of the ITRs range from 200-500bp for variola virus (VARV) to almost 12kbp for vaccinia virus (VACV) strains [7].

Many complete poxvirus genomes have been sequenced [3]. Generally the central region of the genome consists of highly conserved genes involved in essential replication functions (RNA and DNA synthesis, virion assembly, structural proteins) and the terminal regions are more divergent, encoding genes involved in host range, immunomodulation and virulence [1], [3]. The A+T content varies significantly between poxvirus genomes (36-85%) but most poxviruses are A+T rich [8]. Exceptions to this include the parapoxviruses and MCV which each have an average of 36% A+T [9, 10]. There are 90 genes that are common to all ChPV and 49 that are conserved in all poxviruses [3]. The gene content and arrangement within the central genomic region of ChPV is highly conserved, with the exception of species-specific insertions and regions of gene inversion in APVs [3].

Table 1.1 The *Chordopoxvirinae* (ChPV). A list of genera and their respective species [2].

Genus	Species
Orthopoxvirus	vaccinia virus (prototypic species), camelpox virus, cowpox virus, ectromelia virus, monkeypox virus, raccoonpox virus, taterapox virus, variola virus, volepox virus, skunkpox virus (tentative species), uasin gishu disease virus (tentative species)
Parapoxvirus	orf virus (prototypic species), bovine papular stomatitis virus, pseudocowpox virus, squirrel parapoxvirus, parapoxvirus of red deer in new zealand, auzduk disease virus (tentative species), camel contagious ecthyma virus (tentative species), sealpox virus (tentative species), chamois contagious ecthyma virus (tentative species)
Avipoxvirus	fowlpox virus (prototypic species), canarypox virus, juncopox virus, mynahpox virus, pigeonpox virus, psittacinepox virus, quailpox virus, sparrowpox virus, starlingpox virus, turkeypox virus, crowpox virus (tentative species), penguinpox virus (tentative species), peacockpox virus (tentative species)
Capripoxvirus	sheeppox virus (prototypic species), goatpox virus, lumpy skin disease virus
Leporipoxvirus	myxoma virus (prototypic species), hare fibroma virus, rabbit fibroma virus, squirrel fibroma virus
Suipoxvirus	swinepox virus (prototypic species)
Molluscipox virus	molluscum contagiosum virus (prototypic species)
Yatapox virus	yaba monkey tumor virus (prototypic species), tanapox virus
Cervidpoxvirus	deerpox virus
Unassigned	squirrelpox virus

1.1.3 Poxvirus cell entry, replication and morphogenesis

Different forms of infectious poxvirus particles exist which differ with respect to their outer membrane. These include the mature virions (MV) which have a single membrane, the intracellular enveloped virion (IEV) or ~~-w~~rapped virion (WV)" which has undergone additional membrane wrapping and the extracellular (wrapped) virions (EEVs) which are either cell-associated (CEV) or unattached and consist of an MV surrounded by an additional membrane [11].

Poxvirus morphogenesis, as defined by studies on vaccinia virus, is represented diagrammatically in Figure 1.1. Poxvirus infection and replication begins with membrane binding and virion entry into a susceptible cell [12]. Poxvirus entry into cells occurs via two distinct pathways – direct plasma membrane fusion at a neutral pH and endosomal entry at a low pH [1, 11]. Virus entry is facilitated by interaction of viral proteins with cell surface proteins and polysaccharides [13]. Before fusion, the EEV outer membrane must be removed as viral entry-fusion proteins are situated on the MV membrane [1]. The MV fuses with the cell or endosomal membrane during virus entry, depositing the core into the cytoplasm of the cell where gene expression is initiated [11]. After the removal of the MV membrane or both EEV membranes, the viron core enters the cell and is transported via microtubules within the cytoplasm [14]. The cores contain a functional early transcription system and early viral mRNAs are produced within the core itself [1].

The poxvirus replication cycle involves the temporal expression of early, intermediate and late viral genes, involved in each stage of viral morphogenesis and release [1]. Early genes encode mRNAs which are translated to initiate DNA replication, host interactions and intermediate gene expression [1]. In VACV, these genes are divided into immediate- and delayed-early genes [15]. Once DNA replication has commenced, intermediate genes are transcribed which encode regulatory proteins which induce the expression of late genes. Late genes encode the proteins required for the production of progeny virus including structural proteins and the packaged transcriptional enzymes [12].

Assembly of progeny virions occurs in viral factories within the cytoplasm. The first distinct structures are viral crescents which grow to form spherical immature virions (IV) that contain core constituents and the DNA genome [12]. Proteolytic cleavage of core proteins and internal reorganisation transforms the circular IV into a brick-

shaped MV particle. The MV is the most common and abundant form of infectious poxvirus and is released upon cell lysis [1, 12]. Alternatively, virions become enveloped in a double membrane derived from endosomal or trans-Golgi cisterna, producing the aforementioned WV particles which are then released via exocytosis or budding to form the extracellular EV [1, 12].

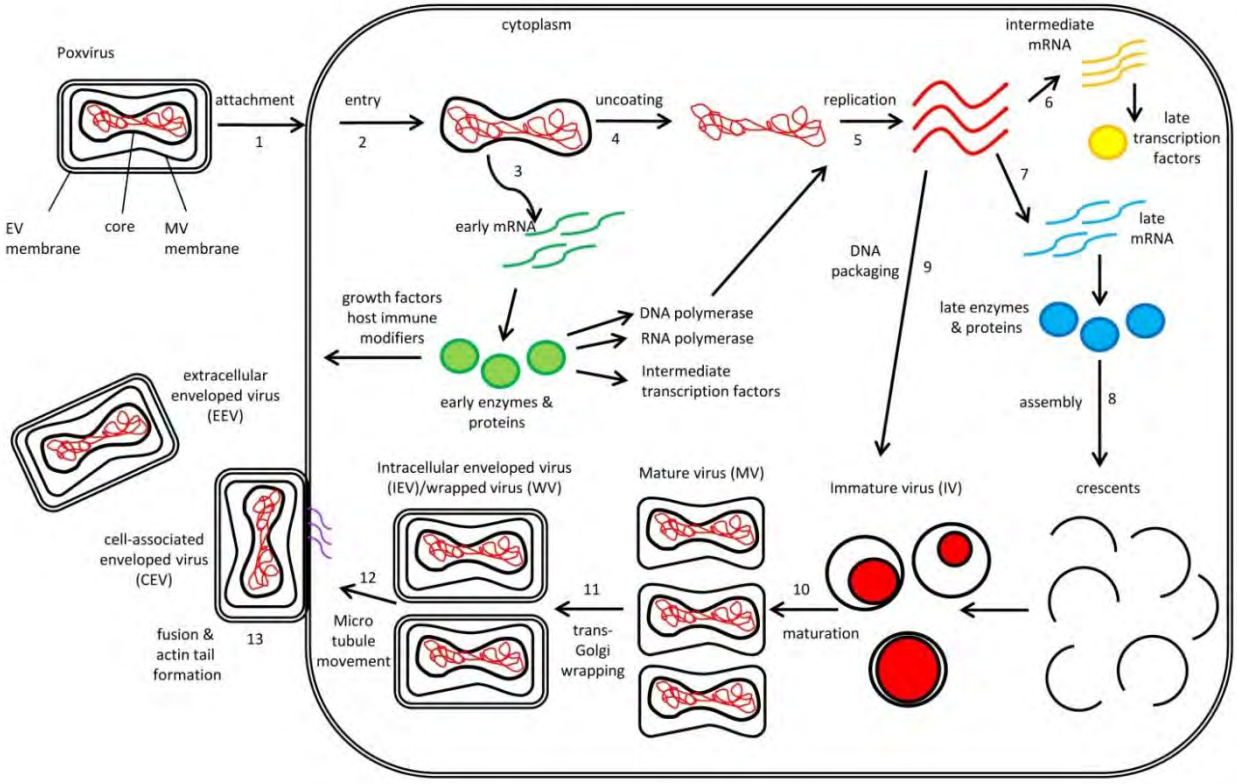


Figure 1.1. Poxvirus morphogenesis. Adapted from Moss, 2007 [1]**.** Major steps in the poxvirus life cycle are represented above and numbered sequentially.

1.2 Avipoxviruses

1.2.1 Avipoxvirus classification and phylogenetics

APVs are divergent members of the ChPV [2] which have been shown to naturally infect more than 278 of the approximately 9000 species of wild and domestic birds [16]. Despite the large number of host species, according to the International Committee on Taxonomy of Viruses (ICTV) there are currently only ten defined- and three tentative APV species [2], with species names originally assigned according to the bird species which they infect or from which they were isolated [17]. Since APV are often not host specific and differ with respect to their virulence the current host-based means of taxonomy and classification has been criticised [18, 19]. An

alternative approach to classification, which takes into account phylogenetic grouping of these viruses, may provide a feasible alternative to the host species-based means of APV taxonomy [18].

APV phylogenetic studies have previously been based on the gene corresponding to VACV P4b (fpv167, VACV A3L) [18-21] which encodes a 75.2 kDa virion core protein, 4b, and is highly conserved amongst all poxviruses [22]. Phylogenetic analysis of this locus indicates that all strains cluster into 3 major clades, A (Fowlpox virus-like), B (Canarypox virus-like) and C (Psittacine). Clades A and B can be further divided into 10 minor clades, namely A1-A7 and B1-B3 [23]. Two additional conserved genes have been used to validate the findings based on P4b, the genes encoding virion envelope protein p35 (fpv140, VACV H3L) [18-20] and VLTF-1 (VACV G8R; fpv126 locus), which encodes the most conserved protein between FWPV and CNPV with 95% amino acid identity [20, 24]. P4b (A3L), VLTF-1 (G8R) and H3L are conserved in all ChPV [3]. It is possible that more robust phylogenetic analyses, such as those based on the concatenated sequences of multiple conserved genes [8], will provide a more accurate means of taxonomy and classification of these viruses.

The most well characterised APVs are the species prototypes, fowlpox virus (FWPV) and canarypox virus (CNPV). Both genomes have been sequenced and found to be considerably divergent, including an average of 47% amino acid divergence between homologues, over 75kbp additional sequence, suggesting that there may be substantial genomic diversity between other APV species [24, 25]. Genetic identities between species within the APV genus are lower (<95%) than the identities between species in other ChPV genera (>96%), suggesting that the APVs may constitute separate genera within the ChPV, or APVs could possibly be classified as a separate subfamily within the Poxviridae [24, 26].

1.2.2 Avipoxvirus genome (CNPV and FWPV)

APV genomes are the largest of all poxviruses, ranging between 260-365kb in length and with low G+C content (30-40%) [25]. Thus far, only three APV genomes have been published; a pathogenic American strain of FWPV (FPVUS) [25], an attenuated European strain of FWPV derived from European FWPV HP1 passaged over 400 times in chick embryo fibroblasts (FP9) [27], and a virulent CNPV isolated from a canary (CNPVATCC VR-111) [24]. FP9, FPVUS and CNPV have large

genomes of 258, 280 and 365kbp encoding 238, 260 and 320 open reading frames (ORFs) respectively [24, 25, 27].

Comparison of the CNPV and FWPV genomes reveals overall synteny in genome arrangement with similar genetic complements. They do, however, exhibit significant differences including rearrangement, insertion and deletion of genes in the terminal, variable regions as well as in three internal, variable regions which is in contrast to the conservation of central genomic regions in other ChPVs. These variable regions within the conserved central region of the genomes occur near the junctions of areas that were identified in FWPV as rearranged relative to other ChPVs and contain genes involved in virus-host interactions [24, 25]. Despite this synteny, amino acid identity between CNPV and FPVUS ORF homologues (55-74%) is similar to that between ChPV genera [24]. The CNPV genome is also 80-100kbp larger than the FWPV genomes [24, 25, 27].

Laidlaw and Skinner (2004) compared virulent (FPVUS (American) and HP1 (European)) and attenuated, tissue culture adapted (FP9 (European)) FWPV strains. Instead of the predicted changes in immunomodulators as the mechanism of attenuation, members of multigene families, especially those encoding ankyrin repeat proteins were seen to be the drivers of attenuation [27]. Ankyrin repeat proteins are thought to be involved in poxvirus host-range [28], and have previously been implicated in the attenuation of sheeppox virus [29]. Over 49% (137 genes) of the CNPV genome and 38% (89 genes) of the FWPV genome are comprised of genes belonging to gene families, 51 and 31 of which are ORFs containing ankyrin repeats respectively [24, 25]. These and other differences in immunomodulatory gene families encoded by APVs may account for the extensive variability in virulence, host range and host interaction [24].

1.2.3 Avipoxvirus pathogenicity

APV-infected birds display various clinical symptoms of poxvirus infection, depending on viral virulence and host susceptibility to the infecting strain. The disease presents in either cutaneous or diptheric forms, whilst non-symptomatic infection is also possible [17]. Cutaneous infection is characterised by nodular, wart-like lesions on the skin, usually in sparsely feathered regions of the body such as the beak, eyelids, legs and combs [17] (Figure 1.2). Diptheric infection presents with proliferative lesions in the upper respiratory and digestive tracts of the infected birds

[17]. These clinical symptoms, however, are sometimes ambiguous and therefore laboratory diagnosis is necessary to determine the causative agent of the condition. Laboratory diagnosis is usually performed by experimental infection of chick chorioallantoic membranes (CAMs), histopathology [30, 31], electron microscopy [32] and/or polymerase chain reaction (PCR) [21].

Figure 1.2 Juvenile Speckled Pigeon displaying cutaneous pox lesions on the eyes and beak. Photograph courtesy of Tertius A.Gous

1.3 Poxviruses as vaccine vectors

1.3.1 History

Historically, poxviruses have been integral to the development and study of vaccines, virology and immunology. In 1748, Dr Edward Jenner discovered that inoculation of humans with cowpox virus (CPV) afforded protection against smallpox, caused by VARV [33-35]. CPV for smallpox vaccination was later replaced with the closely related poxvirus, VACV. These three viruses, although different in their disease causing capabilities, all belong to the genus, Orthopoxvirus, and thus have the ability to cross-protect [35]. In 1980, Smallpox was declared officially eradicated by the Assembly of the World Health Organization (WHO), as a direct result of vaccination with VACV [36]. The year 1980 also saw the application of recombinant DNA technology to VACV, thus enabling the development of genetically engineered expression vectors for vaccination against other diseases [37].

1.3.2 Advantages of poxviruses as vaccine vectors

Poxviruses are undergoing extensive evaluation as vaccine candidates against several pathogens and diseases. There are many advantages of using poxviruses as vaccine vectors. (1) They replicate solely in the cytoplasm of host cells [1], ensuring that no viral DNA integration into the host genome can occur; (2) they are stable as freeze-dried vaccines and can withstand high temperatures [38]; (3) production and administration of poxviruses are relatively inexpensive [37]; (4) poxvirus-based vaccines can be administered via several routes [35] and (5) they can induce both humoral and cell mediated immune responses [39-41]. In addition, (6) poxviruses have large, flexible genomes which can accommodate insertion of up to 25kb [42] of foreign DNA, allowing for the expression of multiple genes within the same vector and allowing for the production of polyvalent vaccines [43].

The use of replication-deficient or host range-restricted poxviruses, such as MVA, NYVAC and APVs, for vaccination confers additional safety advantages. The inability of these poxviruses to complete a full replication cycle and produce infectious progeny in non-permissive cells represents a significant bio-safety advantage [44-48].

1.3.3 Rational design of poxvirus vaccine vectors

Although many vaccine vectors have been tested, there is no one ideal vaccine vector. Therefore, there is a need for the discovery of novel vectors and the development of improved vectors. Rational vaccine design involves defining the correlates of protection and the development of specific vaccines which induce the desired responses [49]. Potential vaccine vector candidates must fulfil specific criteria, which may differ according to the required application. Guidelines set out by the WHO [50] indicate that the vector backbone should not be pathogenic and viruses with specific tropism should be avoided unless otherwise indicated [50]. Non-replicative poxviruses, such as APVs, modified vaccinia Ankara (MVA) and others have considerable safety benefits and may make good vaccine vector candidates in this regard.

Existing immunity to VACV has been shown to negatively influence the immune response elicited upon vaccination with the same or cross-reacting viruses [51]. Furthermore, as seen in the STEP trial testing an adenovirus based candidate HIV

vaccine (Merck rAd5) previous immunity to the vaccine vector may enhance susceptibility to HIV infection in vaccinated subjects [52].

Because of their large genomes and their ability to accommodate large inserts of foreign DNA, poxviruses are promising candidate vectors for rationally designed vaccine strategies [49]. These strategies include the control of transgene expression by poxvirus promoter design, co-expression of cytokines or other immune modulators to enhance immunogenicity, the use of heterologous prime-boost vaccination regimes to enhance target-specific immune responses, targeted deletion of host-range genes to enhance safety and immunogenicity and taking advantage of the early innate immune system by targeting specific innate effector molecules which modulate the subsequent adaptive responses [49].

1.3.4 Vaccinia virus as a vaccine

The OPV, VACV was used in the global program to eradicate smallpox and is the prototype of the poxvirus family. The last recorded natural case of smallpox occurred in Somalia in October 1977 [36]. However, interest in VACV has continued due to its use as a vaccine vector and as a model for the study of virus-host interactions [53]. Although VACV has many attributes that make it an attractive vaccine vector candidate, high incidence rates of complications associated with its use [54] has led to safety concerns and the contraindication of VACV in immunocompromised individuals [37]. Smallpox vaccination of human immunodeficiency virus (HIV) positive individuals has been shown to induce vaccine-associated disease and multiple vaccinations may accelerate HIV-1 progression and acquired immunodeficiency syndrome (AIDS)-related complications [55]. It has also been reported that transmission and dissemination of VACV can occur from vaccinees to individuals in close contact. This contact vaccinia has been known to cause complications and VACV-related adverse events [56]. In order to address these safety concerns a number of attenuated VACV strains have been developed including MVA, defective vaccinia Lister (dVV-L) and NYVAC.

1.3.5 Attenuated poxviruses as vaccine vectors

Highly attenuated, non-replicative poxvirus strains have been developed for use as vaccine vectors which confer a significant safety advantage over infectious strains

[57]. Selected attenuated poxvirus based vaccine candidates in clinical trial for human use are listed in Table 1.2.

Table 1.2 Selected poxvirus based vaccines in clinical development for human use

Recombinant viral vector	clinical trial	Inserts	Targeted Pathogen	Outcome	References
MVA-BN	Phase IIb	-	Smallpox	Protection	[58, 59]
ALVAC containing the gene encoding HIV-1 gp160, and protein gp120	Phase III	gp160 and gp120	HIV-1	Partial protection	[60]
Prime: FP9, Boost : MVA-ME-TRAP	Phase IIb	ME.TRAP	Malaria	No protection	[61]
MVA-5T4 (TroVax) +SOC	Phase III	Trophoblast glycoprotein	Metastatic renal cell carcinoma	Well tolerated. No difference in overall survival	[62]
PANVAC-V/F (rVACV and rFWPV)	Phase III	CEA, MUC1, and TRICOM	Advanced pancreatic cancer	No difference in overall survival	[63, 64]
PROSTVAC-V/F	Phase II/III	PSA-TRICOM	Metastatic castration-resistant prostate cancer.	no change in time to progression, but demonstrated significant survival advantage. Phase III trial in progress	[64, 65]

1.3.5.1 MVA

The highly attenuated MVA was developed by Mayr et al. (1975) after extensive serial passage of the chorioallantoid vaccinia Ankara (CVA) in primary chick embryo fibroblasts [66]. Genomic sequencing revealed that MVA (178kbp) has lost approximately 30kbp of its genome compared to the parental CVA strain [67]. MVA displays abortive growth in human cells and has been shown to be safe even in immunocompromised individuals [68]. It has been found that both early and late viral genes are expressed by MVA in human cells and that viral assembly is inhibited after DNA replication has taken place [69]. Despite the block in virus life cycle MVA has been shown to be highly immunogenic and protective as a vaccine vector against several pathogens, including HIV, malaria and tuberculosis amongst others [70].

1.3.5.2 NYVAC

The attenuated NYVAC strain was derived from the Copenhagen VACV vaccine strain (VACV-COP) by the targeted deletion of 18 ORFs of non-essential genes encoding virulence factors and affecting host range. NYVAC-based vectors have

been found to be safe and immunogenic in humans and animals [44]. NYVAC displays reduced ability to replicate in human cells and is unable to produce infectious progeny virus in humans [44]; however, similar to MVA, it has been shown to be immunogenic and protective against different diseases [70].

1.3.5.3 Avipoxviruses

APVs have a restricted host range and replicate only in birds. Despite this, they have been shown to have the ability to infect non-avian hosts and undergo abortive infection in mammalian cells [45, 71]. Attenuated APVs have been used to vaccinate birds against APV-infection (FWPV, CNPV, quailpox) [26] and recombinant FWPV vectors have protected birds against avian influenza [72], Marek‘s disease virus [73] and Newcastle disease virus [74]. In mammals, both FWPV and CNPV have been used successfully as vaccine vectors for a variety of different pathogens and malignancies, including prostate cancer [75], equine influenza virus [76], rabies [45, 77], canine distemper virus [78] and malaria [48]. APVs are particularly desirable as HIV-1 vaccine vectors as a result of their excellent safety profile and host range specificity [60, 79-84]. The only successful HIV-1 vaccine clinical trial to date has been the RV144 trial, held in Thailand, involving four priming vaccinations with a CNPV (ALVAC) vector expressing HIV-1 gp120/Gag-Pro, followed by two booster injections with a recombinant glycoprotein 120 subunit, AIDSVAX [60]. This trial decreased the rate of HIV-1 infection by 31.2% in vaccinated subjects compared to the placebo group, and although promising, indicates that further optimization of the vaccine is required. This success has stimulated interest in the assessment of other APVs as vaccine vectors.

1.4 Poxvirus-host interactions

The host innate immune system is activated early in viral infection, a response which is important for the activation of adaptive immunity and the subsequent generation of an efficient immune response to virus-encoded proteins. Host-range restricted poxviruses have been shown to successfully activate the host immune system [85, 86]. However evidence exists to indicate that each virus does this in a different way, with an accompanying different pattern of gene expression [87-92]. A recent study has shown that ALVAC, MVA and NYVAC produce distinct innate immune profiles, characterised by different induction of pro-inflammatory and

antiviral cytokines and chemokines in both rhesus monkeys and human peripheral blood mononuclear cells (PBMCs) [93].

1.4.1 Innate immune recognition

The innate immune system is activated when cells sense foreign pathogen associated molecular patterns (PAMPs) through pattern-recognition receptors (PRRs). These include the Toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and the nucleotide-binding and oligomerization domain (Nod)-like receptors (NLRs) [94]. Sensing of viruses by these PRRs activates intracellular signalling pathways that result in the expression of pro-inflammatory cytokines and Type-I interferons (IFNs) which act to limit viral replication and to stimulate the induction of the adaptive immune response [95]. Poxviruses are recognized by a number of different PRRs (summarised in Figure 1.3) with innate immune sensing patterns differing considerably between species and even between different derivatives of the same parent species (VACV, MVA and NYVAC) [96].

The important role played by TLRs in both the innate and adaptive immune responses to pathogens is undisputed. TLRs activate signalling pathways to induce appropriate immune system effector responses. Specifically, TLR3, TLR4, TLR7, TLR8 and TLR9 induce antiviral responses by inducing type I IFN responses [97]. In turn, IFN‘s have been shown to upregulate TLR gene expression in viral infections [98]. Several studies have implicated different TLRs required for innate recognition of poxviruses [99]. Ectromelia virus (ECTV) recognition by murine dendritic cells is TLR9 dependent [100]. Recombinant FWPV is recognised in a TLR7 and TLR9-dependent manner in DCs and both TLRs are important for type I IFN production. [101]. Innate immune recognition of VACV is mediated by TLR2 [99] and TLR2 and TLR6 are important for MVA recognition and subsequent cytokine production [95]. Conversely, TLR3 activation has been shown to contribute to VACV pathogenesis [102] demonstrating that activation of TLRs in poxvirus infection may have a dual role, acting to initiate the host response to infection and as an important mediator of poxvirus pathogenicity.

RLRs such as RIG-I and melanoma differentiation associated gene 5 (MDA5) play an important role in the recognition of cytosolic viral RNA. MDA5 and RIG-I have been shown to be involved in sensing viral RNA species produced by VACV and MVA infection in a cell type dependent manner [95, 103]. In addition, poxviruses

also activate the inflammasome. MVA has been shown to potently activate the NLRP3 inflammasome in the macrophage. The secretion of IL-1β in response to MVA infection requires TLR2 and NLRP3 inflammasome for pro-IL-1b production and caspase-1 activation, respectively [95]

Activation of Inflammasomes during Viral Infection

To date, the NLRP3-, AIM2-, IFI16- and RIG-I-dependent inflammasomes have been shown to be activated during viral infection [104]. Poxviruses have been shown to activate the NLRP3 inflammasome. MVA initiates the NLRP3 inflammasome in human and murine macrophages [95] and myxoma virus (MYXV) activates it in a reactive oxygen species (ROS) and cathepsin B-dependent way [105]. VACV has been shown to be sensed by the AIM2 but not the NLRP3 inflammasome in murine macrophages [106].

1.4.2 Type-I Interferon

The hallmark of the anti-viral response is the production of a type I IFN (IFNα/β) response which can be produced by all nucleated cells in response to viral infection [107]. Studies have revealed that IFN responses are crucial for anti-viral resistance [108]. A number of ~~-c~~lassical" Interferon stimulated genes (ISGs) have been well established, including EIF2AK2 (PKR), MX1, OAS1, APOBEC3G, TRIM5, ZAP, ISG15, ADAR, IFITM1/2/3, BST2 (tetherin) and viperin (RSAD2) [109]. The effect of type I IFNs on poxvirus-induced immune responses is complex. Co-administration of IFN-α with VACV resulted in a decrease in cell mediated immunity whereas IFN-α enhanced antigen-specific T-cell proliferation and cytotoxic T lymphocyte responses when co-administered with FWPV [110]. Conversely, type I IFN is not necessary for the induction of adaptive immunity to FPV-encoded antigen [111]. Taken together, the role that type I IFNs play in the immune response to poxviruses is poorly understood. While type I IFN responses are important for the induction of the immune response to virus infection, they may decrease the immunogenicity of viral-vectored vaccines by promoting anti-viral responses and subsequent viral vector clearance [96]. Therefore a balance is necessary for vaccination purposes, where enough type I IFN needs to be produced to activate the immune system but not so much as to inhibit viral replication and gene expression before antigen presentation can occur [96].

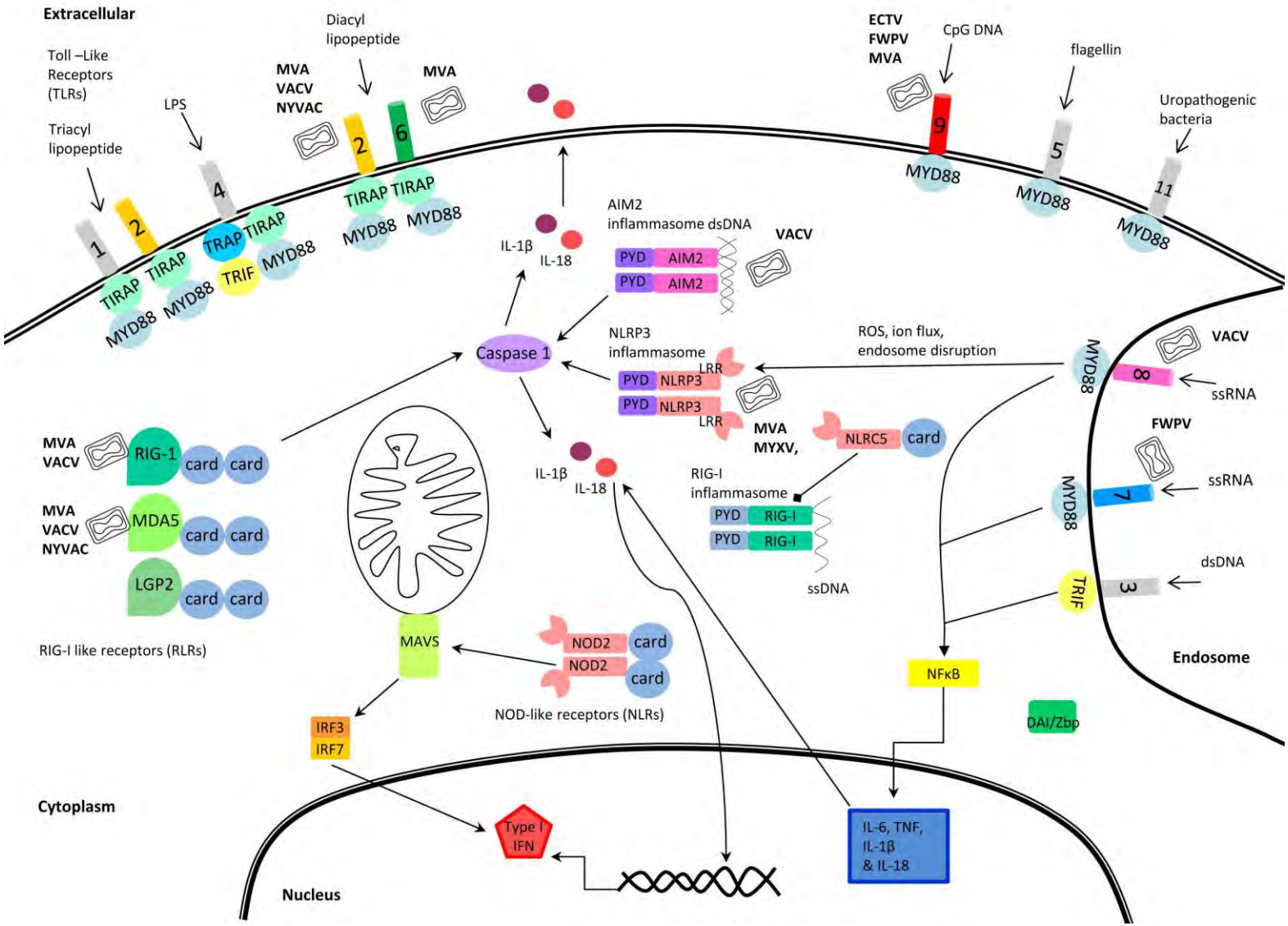


Figure 1.3 Innate immune recognition by poxviruses. Adapted from Kawai et al. 2006 [102] and Kanneganti et al 2010 [117]. Summary of the pattern recognition receptors (PRRs) that are known to be recognised by different poxviruses, including members of the toll-like receptors (TLRs), Retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and inflammasomes.

1.4.3 Poxviruses and cell death

Programmed cell death plays a key role in the innate immune response to viral infection. The mechanism of cell death influences the response of neighbouring cells with different effects on the immune system.

1.4.3.1 Apoptosis

Apoptosis is a form of programmed cell death which eliminates damaged, virally-infected or unwanted cells without disruption of surrounding tissues or the induction of an inflammatory response. The mechanism of apoptosis involves a complex and highly organised energy-dependent, molecular cascade, which can occur via multiple intricate pathways [112, 113]. Apoptosis occurs in two discrete stages; first the formation of apoptotic bodies, followed by their phagocytosis. The formation of apoptotic bodies involves dynamic membrane blebbing [114], cell shrinkage, tight packing of the organelles and both cytoplasmic and nuclear condensation (pyknosis) [115]. In the second stage, these apoptotic bodies are phagocytosed and rapidly degraded [115].

Characteristic biochemical processes take place in cells undergoing apoptosis, including protein cleavage and cross-linking, and DNA degradation [112, 113]. The key players in these events are a set of cysteine proteases or caspases which are proteolytic and cleave proteins at aspartic acid residues. There are ten major caspases; the initiators (caspase 2,-8,-9,-10), the effectors (caspase 3,-6,-7) and the inflammatory caspases (caspase 1,-4,-5) [116, 117]. Several caspase substrates are important in the apoptotic process, and their cleavage results in some of the characteristic molecular and morphological features of an apoptotic cell, including DNA degradation into 180-200bp fragments [118], the expression of surface molecules to ensure rapid phagocytosis and breakdown of the nuclear envelope [119].

The mechanisms of apoptosis involve a complex, energy-dependent cascade of molecular events. Apoptosis typically proceeds through one of two signalling cascades, the extrinsic or death receptor pathway or the intrinsic or mitochondrial pathway. A third pathway involves T cell-mediated cytotoxicity and perforin-granzyme-dependent cell death, via either granzyme A or granzyme B. Extrinsic, intrinsic and granzyme-B-dependent pathways converge on the same execution

pathway involving caspase 3 and -7 [112], whereas the granzyme-A-dependent pathway results in the activation of a caspase-independent pathway [120].

The intrinsic signalling pathway of apoptosis is induced by an array of non-receptor mediated stimuli. These stimuli include negative signals such as the absence of some growth factors, hormones and cytokines, which result in the failure to suppress cell death signals, thus initiating apoptosis. Positive intrinsic pathway stimuli include radiation, toxins, viruses, hypoxia, free radicals and others [112]. In the intrinsic pathway of apoptosis, stimuli induce mitochondrial outer membrane permeabilisation, leading to the release of pro-apoptotic proteins (eg: cytochrome c), from the mitochondrial intermembrane space [121]. This process is tightly regulated by members of the B cell lymphoma 2 (BCL-2) protein family [122].

In the extrinsic pathway, transmembrane death receptor-mediated interactions (FasL/FasR, TNF-α/TNFR1, Apo2L/DR4/5 Apo3L/DR3) transmit pro-apoptotic signals from the cell surface to the intracellular signalling pathways [112]. These receptor/ligand reactions recruit adaptor molecules such as FAS-associated death domain protein (FADD) which bind, dimerize to form the death-inducing signalling complex (DISC) which then activates caspase 8, before proceeding to the execution pathway [112, 123].

The perforin/granzyme pathway of apoptosis is used by cytotoxic T lymphocytes (CTLs) to remove tumor- or virus infected cells. CTLs secrete perforin which forms pores within the membrane of the target cell. Granzymes are then released through the pore and trigger apoptosis in the target cell. Granzyme B cleaves protein within the target cell and is the most potent activator of both caspase-mediated and –independent apoptosis [124]. Granzyme A is unable to activate caspases, but induces single-stranded DNA breaks by cleaving nuclear proteins [125].

Extrinsic, intrinsic and granzyme-B-dependent pathways converge on the same execution pathway involving caspase 3, -6 and -7 as ‗executioner' caspases which activate endonucleases and proteases within the cytoplasm, causing the morphological and biochemical changes characteristic of apoptosis [112].

Viral induction of apoptosis

Viruses can induce apoptosis either directly to assist their own dissemination, or by unintentionally triggering the cellular defense mechanisms that initiate protective

programmed cell death [126]. The induction of apoptosis is an important host defense mechanism in virally infected cells. Apoptosis can eliminate potentially harmful cells via two major mechanisms; the first relies on the recognition of viral peptides and major histocompatibility complexes by CTLs, which then triggers apoptosis in the affected cell. The infected cell can also autonomously sense the abnormal activation of the cell cycle by viral proteins [127] and induce apoptosis itself. The induction of apoptosis in a virally infected cell can, in this way, have a negative effect on virus replication and, in response to this many viruses have evolved mechanisms of controlling the host‘s cell death pathways to suit its own needs [126].

In some instances, the induction of apoptosis is beneficial to the infecting virus. Viruses may actively induce apoptosis at late stages of infection to facilitate dissemination of progeny viruses without initiating an inflammatory and immune response in the host [128]. Virus-induced apoptosis and the subsequent production of membrane-bound apoptotic bodies may play a key role in the egress of non-enveloped, non-lytic progeny viruses from the infected cell, as well as their protection from neutralising antibodies [128, 129]. Several viruses have been reported to produce proteins which induce apoptosis in some way [130]. An example of this is the Hepatitis B protein, pX, which upregulates tumour necrosis factor (TNF) and Myc protein family members and sequesters p53, thereby facilitating the induction of apoptosis [131, 132].

Apoptosis and Vaccines

Virus induced apoptosis is an important consideration when determining the potential of a poxvirus for use as a vaccine vector. If apoptosis occurs too early in the life cycle of the virus, gene expression and antigen presentation will be reduced. Apoptosis is, however, desirable once the transgene of a recombinant vaccine has been expressed, as the formation of apoptotic bodies loaded with antigen and the uptake thereof via the alternative class I antigen presentation pathway (cross-presentation) is important in the induction of an immune response [133]. The mechanisms of apoptosis and its effect on vaccine efficacy are not clearly understood. Both induction and delay/prevention of apoptosis in poxvirus vectors have been shown to improve foreign antigen expression as well as immunogenicity [134-136].

The ALVAC-based HIV-1 vaccine candidate, engineered to express anti-apoptotic PKR inhibitors E3L and K3L from VACV, has been shown to produce reduced levels of apoptosis and enhanced viral antigen production in human cells [134] and is therefore expected to induce enhanced immune responses to HIV antigen compared to the ALVAC-HIV vector without E3L and K3L. In contrast, it has been shown that immunogenicity of CNPV-based vectors is improved by the induction of apoptosis of infected dendritic cells, as a result of the subsequent stimulation of maturation of the surrounding dendritic cells [137].

It would be important to assess any novel vaccine vector candidates for the ability to induce or inhibit apoptosis in permissive and non-permissive cells. This is a potential avenue for the improvement of APV vectors, with the insertion of immunomodulatory genes from poxviruses that infect mammalian hosts.

1.4.3.2 Pyroptosis

Pyroptosis is an inflammatory process that is defined as caspase 1 dependent programmed cell death and is a more recently identified pathway of host cell death in response to pathological stimuli, including microbial infection [138]. Pyroptosis is mediated by caspases and was not initially distinguished from apoptosis [139-142]. However, several important differences distinguish the two processes [138, 143, 144].

One of the main differences between pyroptosis and apoptosis is the mediation of cell death by caspase 1 for pyroptosis [138] and caspases 3, 6 and 8 for apoptosis [138, 141, 145]. In addition, mitochondrial integrity is maintained and cytochrome c is not released during pyroptosis as is the case for apoptosis [145, 146].

Mechanism of pyroptosis

NLRs recognise cytosolic danger signals, triggering a signalling cascade resulting in inflammatory cytokine production [147] and/or caspase 1-activation [148]. The subset of caspase 1-activating NLRs (NALPs, NAIP and IPAF) often act together with TLRs, enhancing susceptibility to caspase 1 activation [138, 149].

NLRs trigger the formation of an oligomeric complex termed the inflammasome [148]. The different inflammasomes are named after the NLR they comprise [150] and are assembled in a pathogen dependent manner [151]. The inflammasome is a

large, multi-protein complex assembled through protein interaction between the caspase recruitment -(CARD) and pyrin domains (PYD) of NLRs and the adaptor apoptosis-associated speck-like protein (ASC), forming a scaffold for pro-caspase 1 recruitment and activation [150].

Activated caspase 1 then serves to mediate processes that facilitate pyroptotic cell death which involves rapid membrane rupture and subsequent release of the cell‘s proinflammatory contents [138]. Caspase 1 induces the formation of a membrane pore which leads to increased osmotic pressure, water influx, cellular swelling and subsequent osmotic lysis of the infected host cell [143]. In addition, DNA cleavage occurs by means of an unidentified caspase 1-activated nuclease, and does not produce the characteristic DNA ladder pattern formed during apoptosis [138, 143, 152]. Accompanying nuclear condensation is observed but, unlike in apoptosis, nuclear integrity is maintained during pyroptosis [153].

Caspase 1

Other than its involvement in pyroptosis, caspase 1 has several important functions including activation of the inflammatory cytokines, Interleukin-1 beta (IL-1β) and Interleukin-18 (IL-18), both of which are important in the response to infection and contribute to the inflammatory response elicited by pyroptotic cells [138, 154]. IL-1β affects a diverse range of innate immune functions including immune cell recruitment, tissue destruction, bone resorption and others [154]. IL-18 also has several roles including the induction of IFNγ and activation of T cells [154].

1.4.4 Poxviral immune evasion strategies

Poxviruses encode a number of host range proteins (Table 1.3) which make use of diverse strategies to modulate several mediators of the host immune response [156]. Without the function of these proteins the virus is more vulnerable to the host immune system. This is demonstrated in the comparison of the two VACV-derived viruses, MVA and NYVAC. The respective arsenals of host range, immunomodulatory genes encoded by the two viruses are largely different, with MVA retaining several genes which target inflammatory responses and NYVAC retaining those which antagonize IFN responses [44, 67]. These differences are reflected in the host responses induced by the two viruses *in vitro,* with MVA inducing a greater type I IFN response and NYVAC inducing a more pro-

Table 1.3. Poxvirus immune evasion genes. Adapted from Seet *et al*. 2003 [153]

Viral protein	Function	Orthopox		Avipox		Leporipox	Capripox	Yatapox	Parapox	Molluscum
		VACV	CXPV	CNPV	FWPV	MYXV	LSDV	YLD	ORF	MCV
Cytokines										
TNF receptor, CrmB	inhibits TNF and LTα	C22L (B28R)	D2L (H3R)	CNPV 086		M002R/L				
TNF receptor, CrmC	inhibits LTα	A53R	A56R							
TNF receptor, CrmE	inhibits TNF		K3R							
TNF receptor, CrmD	inhibits TNF		K2R							
vCD30	TNF receptor family member, binds CD153		C5L							
eIF2α homolog	Inhibition of translation, apoptosis inhibition	K3L	M3L			M156R	LSDV014	12L		
IFN-γ receptor	blocks IFN-γ	B8R	B7R			M007R/L	LSDV008			
IFNα/β receptors	binds type I IFNs and prevents their function	B19R	B17R	CNPV 166	FPV125	M135R	LSDV135	136R		
dsRNA-binding protein	Inhibitor of PKR and 2,5A synthetase, apoptosis inhibition	E3L	F3L			M029L	LSDV034	34L	ORF 20.0L	
IL-1β receptor	blocks IL-1β	B16R	B14R	CNPV 081	FPV125					
Toll-like receptor inhibitor	Signaling inhibition	A46R, A52R	A49R							
IL-18-binding protein	inhibits IL-18		C8L	CNPV 100	FPV073		LSDV015	14L		MC054
Viral IL-10	Immune suppressive			CNPV 018			LSDV005	134R	ORF-IL-10	
OX-2 homolog	Negative signaling of lymphocytes					M141R	LSDV138	141R		
Chemokines										
Chemokine homolog	antagonizes chemokines									MC148R
Chemokine-binding protein	chemokine-binding protein	C23L (B29R)	D1L (H5R)			M001R/L,M 007R/L				
CC chemokine receptor	prevent cc-chemokine binding to their G protein–coupled receptors			CNPV 072,083, 215, 232, 283	FPV060, 061,116, 121		LSDV011	7L, 145R		
Antigen presentation/MHC										
Class I-MHC										MC033L
MHC class 1 heavy chain homolog										MC080R
LAP domain	MHC downregulation					M153R	LSDV010	5L		
MHC class Iα chain-like								2L		
Other poxvirus immunomodulators										
3β-hydroxysteroid dehydrogenase		A44L	A47L	CNPV 063	FPV046			133L		MC152R
Semaphorins		A39R	A41R	CNPV 065	FPV047					
Viral growth factor	regulates cell differentiation and proliferation	C11R	C5R		FPV211	M010L	LSDV016	15L		
Complement inhibition	blocks inflammation	C3L	C17L			M144R	LSDV141	144R		
GM-CSF/IL-2 inhibitor	blocks inflammatory cell migration	A41L	A43L						GIF	
CD47-like protein	integrin associated protein	A38L				M128L	LSDV128	128L		
β-NGF	affects inflammation, cytokine production and cell survival			CNPV 099	FPV072, 076					
Lymphocytic-activating molecule										MC002L

TGF-β	Immune modulation			CNPV 064	FPV080					
VEGF	stimulate cell proliferation and angiogenesis								vVEGF	
Ubiquitin	diverse ubiquitination-mediated cellular functions			CNPV 096						
PYD homologue	Inhibit inflammasome formation by binding ASC; inhibit subsequent caspase-1 activation, and IL-1β and IL-18 production					M13L-PYD				
Anti-apoptotic molecules										
Ankyrin repeat						M005L/R				
Bcl-2 gene family	Bcl-2, apoptosis regulator			CNPV 058	FPV039					
DED domains (vFLIP)	FLIP									MC159R, MC160R
M-T4						M004L/R				
Glutathione peroxidase	Glutathione peroxidase, reducing reactive oxygen species			CNPV087	FPV064					MC066L
Apotosis regulator	regulation of apoptosis					M011L	LSDV017	16L		
M-T2						M002L/R				
RING finger (N1R/p28)	promote virulence by suppressing apoptosis		D7R	CNPV 142,159, 160,165, 168,169, 197,202, 203,205, 207,209, 210,212, 217-222 225-228	FPV075, 124, 150, 155,157,1 59,161, 236	M143R	LSDV140	143R		
SPI-1	Serpin, Protease inhibitors that directly block caspase-1 activation, and IL-1β and IL-18 production	C12L	B20R	CNPV 022,275	FPV010, 204, 251	M152R		149R		
SPI-2/CrmA	Serpin. Protease inhibitors that directly block caspase-1 activation, and IL-1β and IL-18 production	B13R	B12R	CNPV 057	FPV040	M151R	LSDV149	10L		
SPI-3	Serpin. Protease inhibitors that directly block caspase-1 activation, and IL-1β and IL-18 production	K2L	M2L	CNPV 062	FPV044	M008.1L				

***Abbreviations: vaccinia virus (VACV), cowpox virus (CPV) , canarypox virus (CNPV) fowlpox virus (FWPV), myxoma virus (MYXV), lumpy skin disease virus (LSDV), Yaba-like disease (YLD), orf virus (ORF), molluscum contagiosum virus (MCV).**

inflammatory response [87-89]. Furthermore, the removal of additional host range genes from MVA (VACV C6L and K7R) and NYVAC (VACV B8R and B19R) further increased their immunogenicity [157], [158]. Differences in immunomodulatory genes between VACV, MVA and NYVAC are depicted in Table 1.4

Differences in the various proteins encoded by viral vectors, their expression levels and amino acid substitutions which may influence protein-protein interactions, may play a role in the innate immune activation and subsequent adaptive immunity against transgenes. Here we highlight several poxvirus strategies of immune evasion with specific emphasis on innate immunity.

Poxvirus evasion of cell-mediated immunity

Cell-mediated immunity is important for poxvirus clearance [159] and an effective response of this nature requires both innate and adaptive effector cells. Poxviruses have thus evolved mechanisms to reduce the cell-mediated immune response to infection including the down regulation of cell surface receptors involved in antigen presentation and immune cell recognition. MYXV has been shown to induce the down regulation of CD4 expression by an unknown mechanism [160]. Several poxviruses have been shown to decrease expression of major histocompatibility complex (MHC) class I, which are involved in antigen presentation to CD8+ CTLs [161, 162]. This may occur indirectly as a result of poxvirus inhibition of pro-inflammatory cytokines (IFN, TNF) that regulate MHC expression or directly by the action of specific viral proteins, such as the MYXV M153R gene product [163]. Despite protecting from CTL-mediated killing of infected cells, MHC class I down regulation also reduces the MHC class I-dependent signal which prevents killing by natural killer (NK) cells [155]. Presumably to counteract this susceptibility to NK cells, some poxviruses encode decoy MHC class I molecules [164, 165].

Poxvirus inhibition of inflammation, complement, cytokines and chemokines

Following viral infection, an early inflammatory response is initiated to promote immune cell infiltration into the site of infection and to limit viral spread. Poxviruses have evolved several immune modulators which affect multiple aspects of the early anti-viral response networks, including complement factors, cytokines and chemokines [155].

Table 1.4 Immunomodulatory genes in vaccinia virus Copenhagen strain (VACV-C), modified vaccinia Ankara (MVA) and NYVAC. Deleted genes are represented as (deleted); disrupted or non-functional genes are in brackets. Table adapted from [70]

VACV-C	MVA	NYVAC	Function
C23L/B29R	(001L/193 R)	C23L/B29 R	CC chemokine-binding protein
C21L	(deleted)	C21L	binds C3b and C4b and inhibits classical and alternative complement pathways
(deleted)-present in VACV-WR	008L	(C12L)	IL-18 binding protein and inhibits IL-18 induced IFN-γ production
C10L	006L	C10L	IL-1 receptor antagonist (IL-1Ra) homologue.
C7L	018L	(deleted)	Inhibits eIF2-α phosphorylation and apoptosis
C3L	(deleted)	(deleted)	complement 4b binding protein
C2L	(deleted)	(deleted)	Kelch-like protein
N1L	(020L)	(deleted)	Bcl2-like anti-apoptotic protein, contributes to virulence by inhibition of host NF-kappa-B activation in response to pro-inflammatory stimuli such as TNFαa or IL1β
M2L	(deleted)	(deleted)	Prevents phosphorylation of the ERK2 protein and subsequent NF-kB activation
K1L	(022L)	(deleted)	Prevents IκB degradation and so inhibits NF-kB activation
K3L	024L	K3L	decoy eIF2-α which acts as a pseudosubstrate for EIF2AK2/PKR kinase.
F1L	029L	F1L	Protein with a BCL2-like fold, regulates mitochondria-mediated apoptosis
F3L	031L	F3L	Kelch-like protein
E3L	050L	E3L	Binds dsRNA
H1L	091L	H1L	Viral phosphatase that reverses STAT1 activation
H5R	095R	H5R	late transcription factor VLTF-4, phosphorylated by B1R kinase and mediates the inhibition of CD1d1-mediated antigen presentation
A39R	(150R/151 R)	A39R	semaphorin
A40R	152R	A40R	Type II integral membrane protein related to C-type lectins, including NK cell receptors.
A41L	153L	A41L	May interact with several cellular chemokines
A44L	157L	A44L	3β-hidroxysteroid dehydrogenase
A46R	159R	A46R	Targets TLR adaptors inhibiting both MyD88 and TRIF dependent pathways
A52R	(deleted)	A52R	Targets IRAK-2 and TRAF-6 to block the NFkB activation pathway by various TLRs
A53R	(deleted)	(A53R)	TNF receptor homologue
A55R	(deleted)	A55R	Kelch-like protein
B1R	167R	B1R	Virus encoded kinase
B7R	175R	B7R	Affects virus virulence
B8R	(176R)	B8R	IFN-γ binding protein
B13R/B14R	(181R/182 R)	(deleted)	Serine protease inhibitor SPI-2
B15R	183R	B15R	Interaction with host IKBKB and inhibits host NF-kappa-B activation
(deleted)-present in VACV-WR	184R	(B16R)	IL-1β binding protein
B19R	(187R)	B19R	binds IFNα/β
(deleted)-present in VACV-WR	(deleted)	(B22R)	Serine protease inhibitor SPI-1

Poxvirus evasion of complement

Poxviruses encode proteins which modulate or inhibit various aspects of the complement system [166]. VACV encodes a secreted complement control protein (VCP) which inhibits both alternative and classical complement pathways by binding to C3b and C4b proteins [167]. Orthologs of VCP are present in CPV, monkeypox virus (MXPV) and VARV [168]. Poxviruses may also evade complement by retaining host regulators of complement, derived from the host cell membrane, on the EEV outer membrane [169].

Poxvirus evasion of Interferon

The IFN response is an important innate anti-viral defence mechanism and poxviruses have developed several strategies to disrupt the IFN response at different levels. Poxviruses limit IFN production by decreasing the amount of double-stranded RNA (dsRNA) produced during early infection and by encoding proteins that sequester dsRNA species. Poxvirus ORFs and transcriptional termination signals are arranged in such a way as to minimize dsRNA production [170]. VACV gene, E3L encodes a dsRNA-binding protein which inhibits host sensing of dsRNA and the function of IFN-induced anti-virus proteins that require dsRNA binding for activation [170].

Many poxviruses encode IFN-γ-receptor homologs which function as competitive antagonists of IFN-γ [170]. OPV encoded IFN-γ-R homologs bind cross-species IFN-γ whereas MYXV IFN-γ-R, M-T7, binds and inhibits only rabbit IFN-γ [155]. In addition, VACV and other OPVs encode an IFN-α/β receptor (VACV WR B18R) which binds type I IFNs and prevents their function [171, 172].

As well as decreasing IFN induction and blocking IFN interaction with cellular receptors, poxviruses encode intracellular inhibitors of IFN-induced antiviral proteins. Examples of these are the products of VACV genes E3L and K3L [170]. E3L has been shown to block the activity of IFN-induced and dsRNA-activated protein kinase R (PKR) [173]. K3L functions as a decoy of eIF2α, inhibiting phosphorylation of eIF2α and reducing the activation of PKR [174]. E3L and K3L orthologs have been identified in VARV [175] and an E3L-like gene has been found in the parapoxvirus, orf [176].

FWPV is resistant to chicken IFN in chick embryo fibroblasts (CEFs) and FWPV was found to be a poor and late inducer of IFN [177]. Two FWPV ankyrin repeat family

genes have been shown to inhibit chicken Type I IFN: fpv012 blocks the induction of chicken beta IFN whereas fpv014 confers resistance to chicken beta IFN itself [178, 179]. These data identify anti-IFN functions of two members of the ankyrin repeat family of genes, which exist in multiple copies in APV and which have not been previously associated with inhibition of the IFN response [178, 179].

Poxvirus inhibition of chemokines and cytokines

IL-18 induces IFN-γ and other cytokines and chemokines, regulates Th1/2 responses, and can activate NK and cytotoxic T cells [180]. Poxviruses encode IL-18 binding proteins (IL-18BP) which block IL-18 from binding its receptor, so inhibiting its function [155].

TNF is a pro-inflammatory cytokine that is produced by macrophages and activated T cells [181]. Poxviruses encode viral homologs of TNF receptors (TNFR) which bind TNF before cellular TNFR engagement can occur [182].

The Interleukin 1 (IL-1) family of pro-inflammatory cytokines control several aspects of the innate and early inflammatory response [183]. OPVs encode secreted IL-1β receptor decoys (VACV B15R and CXPV B14R) [184]. Furthermore, poxviruses disrupt IL-1 receptor signalling by producing proteins (VACV A46R and A52R) containing the Toll/IL-1 receptor (TIR) domain found in members of the IL-1/ TLRs superfamily of receptors which prevent intracellular IL-1R signalling [185].

The cytokine Interleukin-10 (IL-10) has both immunostimulatory and immunosuppressive functions [186]. Putative orthologues of IL-10 are found in Orf virus, bovine papular stomatitis virus (BPSV) [9], lumpy skin disease virus (LSDV) [187], yaba-Like disease virus (YLDV) [188] and CNPV [24]. The Orf virus IL-10 orthologue has been shown to be immunomodulatory in function [189, 190].

Many poxviruses, including members of the leporipoxvirus and OPV genera encode high affinity cc-chemokine binding proteins, which prevent cc-chemokine binding to their G protein–coupled receptors (GPCRs) [191]. In addition, molluscum contagiosum (MCV) virus gene MC148 encodes an antagonist of cc-chemokine receptor 8 (CCR8) that is expressed by Th2 cells to downregulate Th2-cell mediated immune responses [192].

Most poxviruses encode homologs of cellular growth factors [155]. Epidermal growth factor (EGF) regulates cell differentiation and proliferation by binding to ErbB

receptors [193]. Pox EGF homologs, such as VACV growth factor (VGF), are expressed early to induce proliferation of infected and uninfected cells [155, 194]. The Orf virus encodes a vascular endothelial growth factor (VEGF) homolog which functions to stimulate cell proliferation and angiogenesis [195]. The VEGF homolog plays an important role in Orf virus infection promoting a distinctive pattern of epidermal proliferation and the formation of Orf virus-containing scabs which may contribute to virus transmission [196].

Poxvirus inhibition of cell death

The induction of early apoptosis by the host in response to viral infection would have a detrimental effect on virus replication and production and therefore most viruses have evolved strategies to evade or postpone apoptosis (summarised in Figure 1.4). During their evolution, viruses have acquired genes from hosts or co-infecting pathogens, proteins that interfere with different aspects of the host‘s apoptotic signalling pathways.

Caspases play a key role in the apoptosis process, and viruses have developed several ways of interfering with their activity. In CPV, the serine protease inhibitor, cytokine response modifier A (CrmA), is a specific inhibitor of caspases [197] and inhibits apoptosis induced in response to Fas ligand interactions, TNF-α and CTLs [198-200]. CrmA was first established as an inhibitor of caspase 1 [201], but can also inhibit CTL-induced granzyme B as well as caspase 8 and 10 [202]. The CrmA homolog, VACV B13R (SPI-2), inhibits apoptosis in a similar way but is generally less potent than CPV CrmA [203]. In addition to serpins, MCV inhibits caspase 8 activation by producing two FLICE/caspase 8 inhibitory proteins (vFLIPs) (MC159, MC160) that bind to Fas-associated death domain (FADD) and pro-caspase 8 and inhibit apoptotic signals [204].

The conserved family of pro- and anti-apoptotic Bcl-2 proteins are responsible for the regulation of apoptosis at the mitochondrion [205]. Several viruses encode Bcl-2 homologs which inhibit the progression of apoptosis by inhibiting the release of cytochrome c at the mitochondria [206]. FWPV encodes a Bcl-2 homologue (fpv039) that neutralizes the pro-apoptotic Bcl-2 family member, Bak [207].

In addition to Bcl-2, some poxviruses encode proteins that localize to the mitochondrion and inhibit apoptosis, but are not homologous to Bcl-2 proteins. VACV encodes a gene, F1L, which lacks homology to Bcl-2 but inhibits apoptosis at

the mitochondria by constitutively interacting with Bak [208, 209]. Similarly, Myxoma virus encodes the gene, M11L, which interacts with Bak to inhibit apoptosis [210][211]. Orthologues of F1L are found only in other OPV, and M11L orthologues in leporipoxviruses, capripoxviruses, suipoxviruses and yatapoxviruses [207].

Oxidative stress induced by viral infection can cause apoptosis, and can be produced by macrophages or neutrophils. MCV gene MC066L encodes a selenocysteine which functions as a glutathione peroxidase, reducing ROS in infected tissue and preventing apoptosis [212]. APVs, FWPV and CNPV, encode glutathione peroxidase homologs (fpv064 and cnpv087) [24, 25] which may have a similar function.

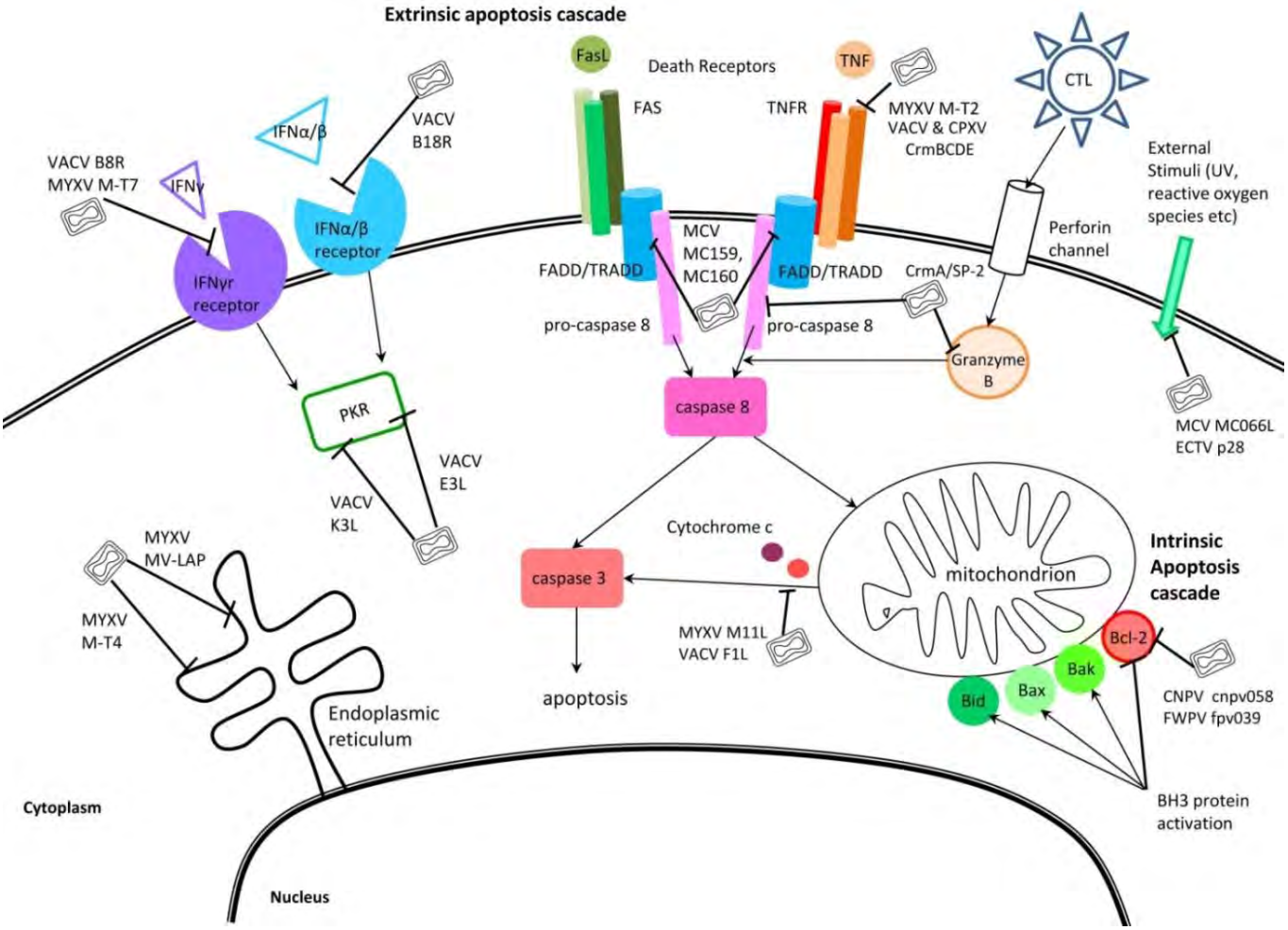


Figure 1.4 Poxvirus modulation of apoptosis. Figure adapted from Taylor et al, 2006, Everett et al. 2002 and Turner et al. 1998 [219-221]. A summary of the known poxvirus strategies to counteract apoptosis. (MYXV = myxoma virus, CNPV = canarypox virus, FWPV=fowlpox virus, VACV = vaccinia virus, MCV = molluscum contagiosum virus)

1.5 Project rationale and aims

There is a need for the discovery of novel vaccine vectors for many different antigens. Repeated use of the same vector for vaccination against different diseases can be limited by the induction of anti-vector immunity, which in some cases may diminish the response to the foreign gene being expressed [213-217]. Vaccination strategies against most HIV-1, have shifted towards the use of prime-boost regimens, often with the use of heterologous vaccine platforms [60, 218, 219]. For HIV, it is anticipated that even a successful vaccine will require multiple boosts throughout an individual‘s life to maintain effective immunity, and it may be necessary for these booster vaccines to express different HIV-1 antigen genes. There is thus an important requirement for novel vaccine vectors. APVs make attractive candidates for use in this regard, as these viruses are highly divergent and limited cross-protection occurs between isolates from different clades and sub-clades [220-222].

None of the APVs isolated in Africa have been developed as vaccine vectors. APVs can be important pathogens of birds and to date very little work has been done on any aspect of these viruses in Africa. APVs can be used as vaccine vectors for birds, mammals and humans alike. Considerable success has been demonstrated using the canarypox virus ALVAC as a vaccine vector [47, 60, 75, 76]. However there is room for improvement of the APV vaccine vectors and it is possible that novel APVs may make better vaccine vectors for certain diseases than the established ones being used, namely FWPV and CNPV.

We hypothesise that there are novel South African APVs that could be developed as vaccine vectors.

Aim 1: The preliminary characterization and epidemiology of 8 novel South African Avipoxviruses to select one isolate for more in depth analysis.

a. Growth and histopathological characterisation of 8 South African avipoxviruses on chick CAMs.
b. Phylogenetic analysis of these avipoxviruses using genetic loci corresponding to fpv 26 (VLTF-1), fpv167 (P4b), fpv140 (H3L) and fpv175-176 (VACV A11R-A12L).

Aim 2: Further characterization of a selected virus to assess its suitability for use as a potential vaccine vector.

a. Analysis of the growth and morphogenesis of the selected avipoxvirus in permissive and non-permissive cells.
b. Analysis of apoptosis induced/inhibited by selected avipoxviruses.

Aim 3: Whole genome sequence analysis of a South African avipoxvirus.

- Extraction and purification of DNA from the selected avipoxvirus.
- Sequencing of the selected avipoxvirus genome.
- Annotation of both the nucleic acid and putative amino acid avipoxvirus sequences and comparison with published avipoxvirus sequences (FPVUS, CNPV-ATCC, FP9).

Aim 4: Assessment of early host responses induced by different poxviruses in mice by transcriptomic profiling.

- Extraction of total (viral and cellular) RNA from infected and mock-infected mouse spleens and microarray analysis of this RNA.
- Analysis of gene profiling data and comparison of the responses induced by the different viruses.

Chapter 2

Basic characterisation of novel South African avipoxviruses

2.1 Introduction

The aim of this study was to characterise some of the APV isolates circulating in South Africa. This will allow us to select one isolate for more in depth analysis. Relatively little information is available regarding the APV strains circulating in South African (SA) birds. APV infection of an African Penguin (*Spheniscus demersus*) [20, 71], a Flamingo (*Phoenicopterus minor*) [223], Ostriches (*Struthio camelus australis)* [224], Cape Turtle Doves (*Streptopelia capicola*) [225] and a Cape Thrush (*Turdus olivaceus*) [225] have been described.

Poxviruses can be grown on the nutrient rich CAMs of embryonated hens' eggs [71, 226, 227] which provide ideal conditions for high titre viral growth. Poxvirus growth on CAMs produce raised, circular lesions, or ~~p~~ocks" with variable morphology dependent on several factors, including the strain of infecting virus and conditions of growth. Most work describing the differences in poxvirus growth characteristics has been done on OPV [228-232]. However, Manarolla et al. (2010) [19] have described differences in gross lesions, membrane thickening and histopathology of 15 APV from Northern Italy and a recent study in Egypt described the gross pock morphologies of 7 APV isolates [233]. Case reports have also described the growth characteristics of individual APV isolates [234-237]. It has been shown that different phenotypes or growth characteristics may influence the immune response in the CAM tissue [231, 238].

A thorough comparison of the macroscopic and histopathological growth characteristics in the CAM model will help to differentiate between several closely related APVs, which, according to phylogenetic analysis of conserved genetic regions, are indistinguishable. In addition, the comparison of the immune cell infiltration and inflammation in the CAMs infected by different viruses may provide a means of predicting which viruses would induce better immune responses in other *in vivo* models and will help to inform the choice of one or more APVs for further characterisation as a potential vaccine vector.

APV phylogenetic studies have previously been based on the genes corresponding to VACV P4b (fpv167, VACV A3L) [18-21], virion envelope protein p35 (fpv140, VACV H3L [18-20] and VLTF-1 (VACV G8R; fpv126 locus) [20] (See chapter 1, section 1.2.1). APVs cluster into 3 major clades, A (Fowlpox (FWPV)-like), B

(Canarypox (CNPV)-like) and C (Psittacine). Clades A and B can be further divided into 10 minor clades, namely A1-A7 and B1-B3 [23].

Members of the *Columbidae* family, consisting of pigeons and doves are susceptible to APV infection and cases have been reported in several Columbiform species including the Mourning Dove [239], Laughing Dove [240], White-tailed Laurel Pigeon [241], Rock Pigeon [18] and others [242]. Poxvirus infection of Columbiformes causes up to 90% morbidity, however mortality is usually limited [243]. Due to the host species based approach to APV taxonomy poxviruses infecting the Columbiformes are designated as pigeonpox viruses. Phylogenetic analysis has indicated that different ~~p~~pigeonpox" viruses group independently and pigeonpox virus isolates have been shown to group in clade A (subclade A2 according to analysis of the P4b gene [18, 244], or subclade A3 according to the H3L gene [18, 233, 244]) as well as in clade B, subclades B1 and B2 [18, 19, 245]. The Columbiformes are therefore susceptible to several different strains of APV and the species designation ~~p~~pigeonpox" does not account for this variation.

In this study, 8 novel SA APVs have been isolated from pigeons and doves (members of the family *Columbidae*, order Columbiformes) from different locations in SA (Table 2.1). These isolates were characterised and compared to FWPV and CNPV (Table 2.1) in terms of their growth on CAMs. This is a comparative study of the macroscopic and histopathological characteristics of 11 APV isolates and is the first of its kind in sub-Saharan Africa. To determine the divergence of the APVs, phylogenetic analysis has been performed based on the 3 previously published loci corresponding to *fpv*167 (P4b), *fpv* 26 (VLTF-1) and *fpv*140 (H3L), as well as an additional locus corresponding to *fpv*175-176 (VACV A11R-A12L) [246]. FWPV ORFs 175-176 are orthologs of conserved VACV A11R and A12L, which encode a non-structural protein involved in virion formation [247] and a 25kDa core protein involved in multiple stages of morphogenesis [248], respectively.

This is the first investigation on what species of APV are circulating in SA birds. Furthermore, the comparison of the growth and morphology of different SA APV will be the first to thoroughly compare the macroscopic, microscopic and histopathological characteristics of several isolates simultaneously. This data will allow us to choose one virus for further analysis.

2.2 Materials and methods

2.2.1 Virus isolates

Avipoxviruses were isolated from lesions on infected birds obtained from various sources in South Africa (Table 2.1). Small sections of approximately 2mm^2 were aseptically separated using sterile surgical blades, homogenized in phosphate buffered saline (PBS) containing penicillin, streptomycin and fungin (PSF) (appendix 1) using a Tenbrook grinder and centrifuged at 14000rpm (Eppendorf Centrifuge 5417C) for 5 minutes. The supernatant fluid, containing virus, was collected and used for growth on CAMs of 10-11 day old fertilized hens' eggs.

Wild-type CNPV was obtained from Prof. K. Dumbell's collection housed at the University of Cape Town and was originally from Prof. A. Mayr (Veterinary Faculty, University of Munich, Munich, Germany). The FWPV vaccine is DCEP 25 modified strain (Sanofi Merial, Duluth, GA, USA) obtained from a licensed veterinarian.

Table 2.1. List of avipoxvirus isolates used in this study.

Virus Abbreviation	Host Species	Symptoms	Geographical source	Date of Isolation
RP2	Rock pigeon *Columba guinea*	unknown	Claremont, Cape Town, W. Cape	1993
LD2	Laughing Dove *Spilopelia senegalensis*	Small diptheric lesion in the lower beak.	Port Elizabeth, Walmer*, E. Cape	2011
Pi5	Racing Pigeon *Columba livia domestica*	Lesion around the eye	Pineview, Grabouw, W.Cape	2011
FeP2	Feral pigeon *Columba livia*	Lesions around the eyes	Port Elizabeth*, E. Cape	2011
LD1	Laughing Dove *Spilopelia senegalensis*	unknown	Table View†, Cape Town, W. Cape	June 2009
FeP1	Feral pigeon *Columba livia*	Lesions around the eyes	Port Elizabeth, Richmond Hill*	2011
RP1	Rock Pigeon *Columba guinea*	unknown	Table View†, Cape Town, W. Cape	20 March 2010
SP1	Juvenile Rock pigeon (Speckled) *Columba guinea*	Severe lesions on the beak and eyes	Table View†, Cape Town, W. Cape	1 February 2010
FWPV Fowl pox virus, (DCEP 25 modified strain (Merial))	Fowl *Gallus gallus*	unknown	attenuated for commercial use	unknown
CNPV	Canary *Serinus canaria*	unknown	Unknown#	unknown

*. Dr Peter Kroon: Southern Cross Veterinary Clinic
†. SANCCOB: Southern African Foundation for the Conservation of Coastal Birds
#. From Dumbell collection, originally from Mayr

2.2.2 Virus growth and titration on CAMs

Virus isolates were grown and titrated on the CAMs of two species of embryonated 10-11 day old chicken eggs using a method described by Joklik (1962) [227], Stannard, et al. (1998) [71] and Kotwal & Abrahams (2004) [226]. Specific pathogen–free (SPF) White Leghorn eggs were obtained from Avifarms (Pty) Ltd (Lyttelton, South Africa) and healthy Cobb Avian 48 eggs were obtained from a commercial company; health status of the layers was checked by an experienced veterinarian.

The eggs were swabbed with 70% ethanol and candled to locate their air sac and blood vessels. Using an egg pricker, small holes were made both at the air sac and on the vascular dorsal region of each egg (Fig. 2.1). A drop of PBS + PSF (appendix 1), was placed on the dorsal opening and a pipette bulb was used to create a vacuum, producing a new air sac on the dorsal side of the egg (see figure). The eggs were inoculated with 100ul virus/egg, using a Tuberculin syringe, into the dorsal air sac, between the CAM and the shell before gentle rotation and incubation at 37°C for 4-5 days. The chicks were killed, either by placing at 4°C for >4hrs or by decapitation, and the CAMs were harvested. Membranes were rinsed in physiological saline and shaken in glass Universal bottles containing glass beads with 1ml McIlvains buffer + 20% Vertrel ® (1,1,1,2,3,4,4,5,5,5-decafluoropentane) (DuPont, Wilmington, DE, USA) per membrane. Thereafter they were centrifuged (Sigma 1-15) at 800rpm for 10mins and the supernatant fluid was centrifuged at 3000rpm for 30mins. The resulting supernatant fluid was transferred to a gas sterilized 50ml Oakridge style Nalgene ® tube (Sigma Aldrich, St. Louis, MO, USA) and a sucrose cushion consisting of a 36% Sucrose solution in TE buffer pH9 (appendix 1) was added to the base of each tube. This was then centrifuged at 11000rpm for 1hr at 4°C in a Sorvall RC5C Plus centrifuge and Sorvall S534 rotor. For further purification, the resulting resuspended viral pellet was centrifuged through a gradient of 36% sucrose and 10% Dextran (in TE buffer pH 9) at 11000rpm for 1hr at 4°C and resuspended in TE buffer (pH 9).

To titrate virus stocks, serial dilutions made in PBS + PSF (appendix 1), were inoculated onto CAMs in triplicate as described and incubated at 37°C for four days. Thereafter, the membranes were spread out on petri dishes and the average number of pocks per dilution was determined.

The pock forming units per millilitre (pfu/ml) was determined by the following equation:

[average number of pocks] x [dilution factor] x 10 = titre (pfu/ml).

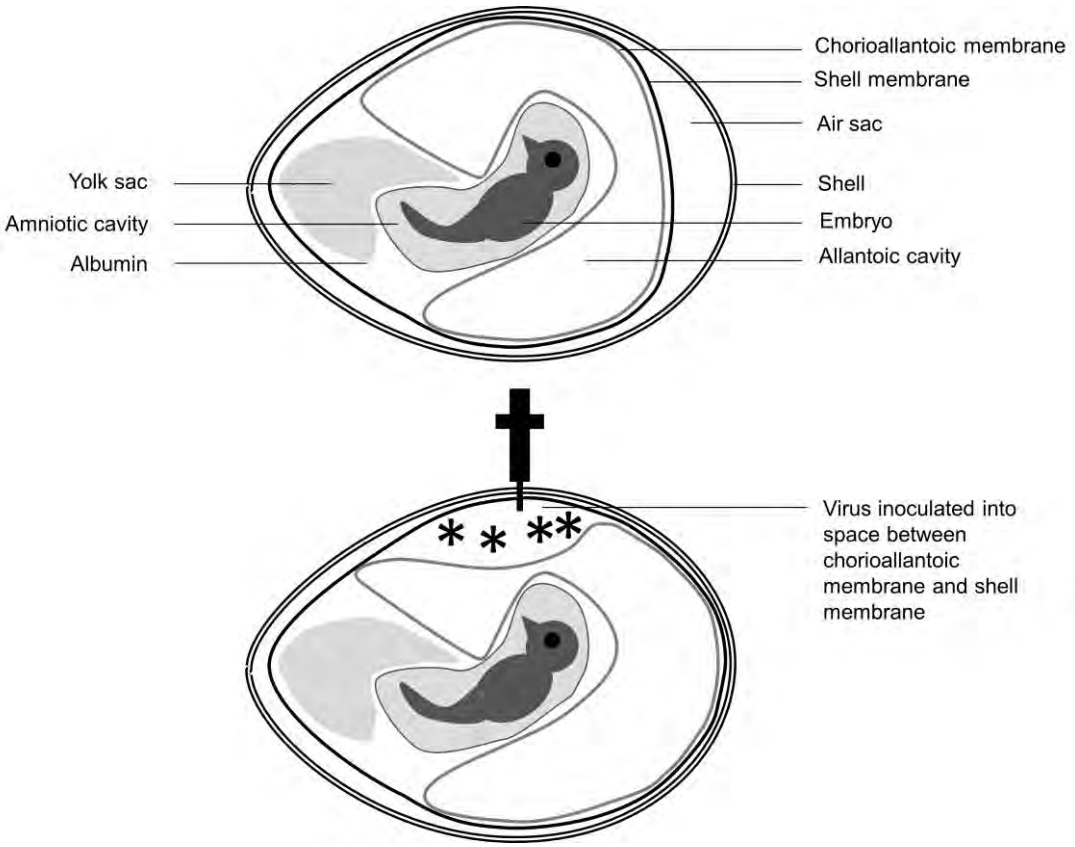


Figure 2.1. Diagrammatic representation of the anatomy of an embryonated hen's egg.

2.2.3 Histopathology

For histopathological analysis, each virus was inoculated onto the CAMs of three 10-day-old commercial Cobb Avian 48 eggs at a constant titre of 10^3 pfu per egg and incubated for 5 days at 37°C before harvesting as above. A titre of 10^3 pfu per egg was chosen for analysis as it gave a good indication of virus growth differences on CAMs. Higher titres were seen to be pathogenic to the chicks, and lower titres did not produce confluent layers of viral growth on the membranes. Determination of gross pathology of the virally-infected CAMs was performed multiple times on different batches of eggs, and the growth characteristics of the respective viruses

did not differ. Harvested membranes were photographed and a representative membrane was chosen for histopathology analysis of each virus. The membranes were fixed in 10% buffered formalin (appendix 1). Infected portions of tissue with similar pock densities were chosen and cut for histopathology. These were rolled up to include multiple pocks on a large surface area, and embedded in paraffin. The samples were cut into 4 mm^2 sections and stained with haematoxylin and eosin (HE) (courtesy of Dr Ross Millin and Ms Anna Marie Beukes, Pathcare). Slides were examined and photographed using a light microscope. Results were confirmed with a specialist avian pathologist, Dr Tertius Gous.

2.2.4 PCR amplification and phylogenetic analysis

Extraction of viral DNA for PCR

Viral DNA was extracted from virus which had been subjected to one high-speed spin through a sucrose cushion. Proteinase K was added to the virus preparation at 2mg/ml and incubated at 55°C for 30mins. An equal volume of lysis buffer (appendix 1) was then added before further incubation at 55°C overnight. An equal volume of phenol:chloroform (1:1) was added and the mixture was inverted and centrifuged at 14000rpm (Eppendorf Centrifuge 5417C) for 5mins. The aqueous phase was removed and incubated with RNAse (100ug/ml) at 37C for 1hr. Thereafter conventional phenol/chloroform extraction followed by sodium acetate and ethanol precipitation was performed [249]

Polymerase Chain Reaction

PCR was performed for the P4b and H3L loci using previously described primers [18]. For VLTF-1 and fpv175-176 (VACV A11R-A12L), the following primers were used to amplify 700bp products for both regions:

VLTF-1 Forward Primer: 5‘-TAAATGAGTTTGCGTATAAAAATCGATAAG-3‘
VLTF-1 Reverse Primer: 5‘-TTCAGCATCCATAACTATCTTTGACTC-3‘
fpv175-176 Forward Primer: 5'-GGTACCGTATATTTCTATAAAACAATATCAC-3'
fpv175-176 Reverse Primer: 5'-ACTAGTGCTAAATCATATTAATGCTATTACGG-3'

A 2x PCR reaction mix, Immomix™ (Bioline, Taunton USA) was used according to manufacturer‘s instructions and PCR thermocycling was performed in a GeneAmp® PCR system (Applied Biosystems, Carlsbad USA). Amplicons were purified using a commercial kit (Zymo research DNA clean and concentrate™-25, Irvine USA), and

sequenced using the BigDye Teminator V3.1 sequencing kit (Applied Biosystems, Carlsbad USA) using an ABI3130xl sequencer (Applied Biosystems, Carlsbad USA) by the University of Stellenbosch Central Analytical Facility.

Sequence and phylogenetic analysis

Sequence analysis was performed using CLC Bio Main Workbench software and MEGA5 [250]. Appropriate models for each data set were tested using MEGA5 and Maximum Likelihood trees were constructed based on MUSCLE nucleotide alignments of P4b, VLTF-1, H3l, and fpv175-176. Maximum likelihood (ML) trees were constructed using the Tamura 3-parameter model with gamma distrubution [251], with a bootstrap test of 100 replicate samples.

2.3 Results

2.3.1 Gross pathological and histopathological characterisation of avipoxviruses in South Africa

Nine out of the 10 APV isolates (Table 2.1) were analysed in terms of their growth and histopathology on CAMs. SP1 (Speckled Pigeon) caused no visible pocks on CAMs and was excluded.

The 9 APV isolates could be divided into five groups based on pock and CAM morphology (Table 2.2). CNPV (Canary) caused no obvious membrane thickening and resulted in small, yellow pocks (Fig 2.2). FWPV caused haemmorhagic pocks, RP2 (Rock Pigeon), LD2 (Laughing Dove) and Pi5 (Racing Pigeon) caused slight thickening of the CAM (Fig. 2.2). RP2 and Pi5 presented white pocks which were variable in size with some pocks having slightly hemorrhagic centres (Fig. 2.2). FeP2 (Feral Pigeon) and LD1 (Laughing Dove) caused a substantial amount of membrane thickening (Fig. 2.2). The pocks resulting from FeP2 and LD1 infection were white and variable in size. FeP1 (Feral Pigeon) and RP1 (Rock Pigeon) caused such extreme membrane thickening that individual pocks were not visible (Fig. 2.2).

Histopathology of these virally-infected CAMs revealed significant differences (Fig. 2.2 and Table 2.2). Although viruses which caused severe macroscopic proliferation of the CAM were noted to have extensive mesodermal hyperplasia and less

epidermal hyperplasia (Fig. 2.2), a more detailed histological analysis showed all viruses to be different from one another (Table 2.2).

All infected CAMs showed varying degrees of hyperplasia and hypertrophy of both epidermal and mesodermal cells. Infected tissue exhibited ballooning degeneration of keratinocytes, necrosis and large, eosinophilic, intra-cytoplasmic inclusions which are the Bollinger bodies described in poxvirus infections [30, 252] (Fig. 2.3). Varying degrees of heterophil and lymphocyte infiltration were most notably observed in the mesoderm and to a lesser degree in the epidermis of the infected membranes. The viruses FeP2 (Feral Pigeon), Pi5 (Racing Pigeon) and LD2 (Laughing Dove) exhibited pronounced immune infiltration and angiogenesis was seen in the mesoderm (Fig. 2.3). Hyperkeratosis and vacuolisation was noted in CAMs infected with RP1 (Table 2.3). Hyperplastic epithelial nests were noted in the mesoderm of FeP2 and FeP1 (Table 2.3). Angiogenesis and fibroplasia were observed to varying degrees in most isolates (Table 2.3).

Table 2.2. Growth characteristics of avipoxviruses

Grouping according to pock and CAM morphology	Virus	Country of Origin	Membrane Thickening	Pock Morphology
1	CNPV	unknown	none	small distinct yellow pocks
2	FWPVvac Fowl pox virus, (DCEP 25 modified strain (Merial))	unknown	slight thickening of CAM	variable size, haemorrhagic/red pocks
3	RP2 (PGPV93K RP2)	SA	slight thickening of CAM	variable size white pocks
3	LD2 (PGPV11K LD2)	SA	slight thickening of CAM	variable size white pocks
3	Pi5 (PGPV11K Pi5)	SA	slight thickening of CAM	variable size white pocks
4	FeP2 (PGPV11K FP2)	SA	substantial thickening of CAM	variable size white still visible
4	LD1 (PGPV10K LD1)	SA	substantial thickening of CAM	variable size white still visible
5	FeP1 (PGPV11K FP1)	SA	severe thickening of CAM	no individual pocks visible
5	RP1 (PGPV10K RP1)	SA	severe thickening of CAM	no individual pocks visible
unassigned	SP1 (PGPV10K SP1)	SA	-	-

Table 2.3. Histopathological comparison of 9 Avipoxviruses

Virus	Macroscopic Thickening of membrane	Epithelial Hyperplasia		Mesodermal hyperplasia/ oedema	Angiogenesis	Fibroplasia	Inclusions	vacuolisation	Ballooning degeneration	Sloughing	Necrosis	Immune cell infiltration		Additional comments
		Chorionic epithelium	Allantoic epithelium									lymphocytes	Heterophils	
FWPV	++	++++	+++	++++	++++	+	+++	++++	++++	++++	+++	+++	++	Significant angiogenesis and blood vessels full of red blood cells.
CNPV	+	++	+/ +++	++	+	+++	++	++	++	++	+++	++	+	Focal necrotic or keratinaceous crusts, Focal areas of fibroplasia and chorionic epithelial hyperplasia, Mesodermal and perivascular infiltration of lymphocytes/plasma cells
RP2	++	+++	++	+++	+++	+	-	++	++	+++	+++	++	++	Granulocyte/heterophill infiltration in epidermis with necrosis
LD2	++/+++	+	+	++	+++	++	+	-	+	+	+	+++	+++	
Pi5	++	++	++	++	+++	+	+++	+++	+	-	-	+++	+++	Formation of vesicles that are not see in others. Infected cells lyse and then fuse to form a vesicle. “Clefting” Vesicles mostly clear with a few granulocytes and epithelial cells. Leucostasis of blood vessels
FeP2	+++	+++	++/+++	++++	+++	+++	+++	++	++	++	++	+++	+	Hyperplastic epithelial nests within mesodermal tissue. Papilliform projections of allantoic epithelium. Pale inclusions indicative of a higher lipid content. Angiogenesis of surface capilliaries and leucostasis
LD1	+++	++	+	+++	+++	+/++	+	-	+++	++	+	+++	+	Areas of severe ballooning degeneration of epithelial cells. Focal areas of heterophil and lymphocyte infiltration and fibroplasia in mesoderm. Leukostasis
FeP1	++++	+++	++	++++	+	++	++	-	+	+	+	+++	++	Pale inclusions indicative of a higher lipid content. Epithelial nests within mesodermal tissue
RP1	++++	++	+	+++	+++	+++	++	+	+	-	+	+++	+	Pale inclusions. Beginning of vacuolisation. Fibroplasia and angiogenesis in mesoderm just below chorionic epithelium

\+ little, ++ moderate, +++ extensive, ++++ extreme case, / both instances present, - None visible

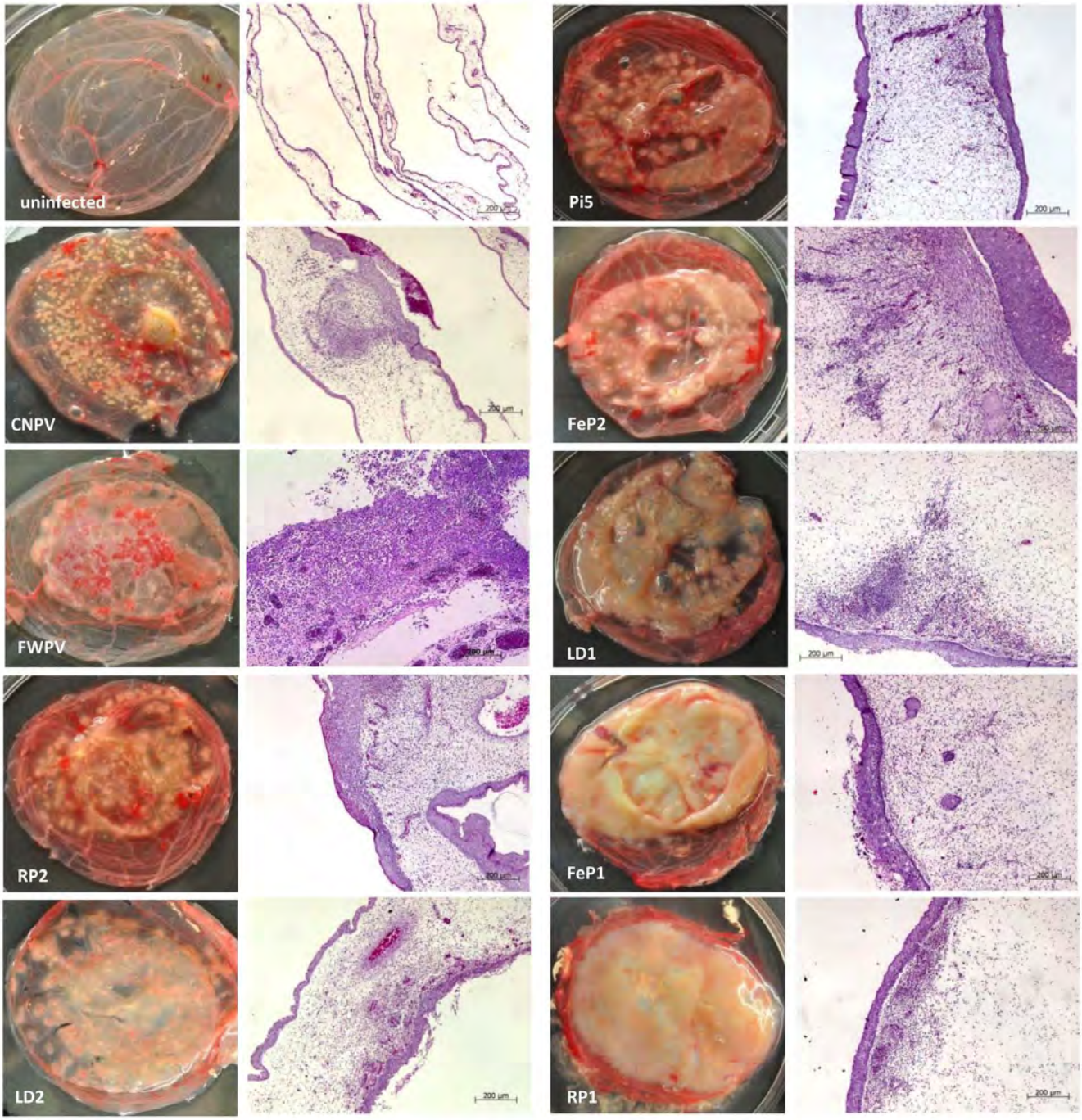


Figure 2.2 Macroscopic and histological comparison of uninfected and infected chorioallantoic membranes of embryonated chicken eggs. 10^3 pfu of each virus were inoculated onto the CAMs of 10-11 day old embryonated hens' eggs. Differences in pock morphology and degree of inflammation of the CAM tissue were observed. Other observations have been tabulated in Table 3. Histology: 10x, H & E stain, Scale bar = 200µm. Virus abbreviations are shown in Table 1.

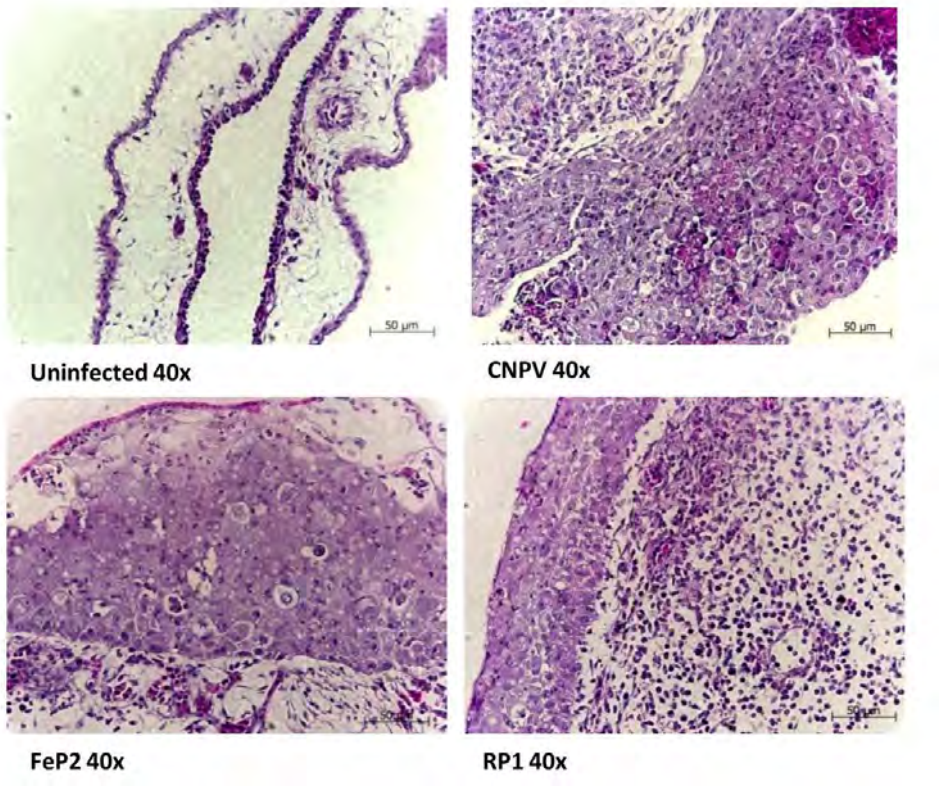


Figure 2.3 High magnification comparison of uninfected CAM and CAM infected with 10^3 pfu CNPV, FeP2 and RP1. Histology: 40x, H & E stain, Scale bar = 50µm

2.3.2 The phylogenetic analysis of avipoxviruses in South Africa.

Nucleotide and amino acid sequences corresponding to fpv 26 (VLTF-1), fpv167 (P4b), fpv140 (H3L) and fpv175-176 (VACV A11R-A12L) were aligned with published sequences obtained from Genbank (See annotations for accession numbers) and phylogenetic relationships were determined based on these alignments. Because of the highly conserved nature of the genes analysed, nucleotide sequences rather than amino acid sequences were used to determine divergence [18, 20]. Clades and subclades have been named according to previous APV phylogenetic studies based on the P4b gene locus [18, 23]

P4b (VACV A3L; fpv 167 locus).

The P4b gene was amplified by PCR and gave the expected 578bp product for all 8 of the SA APV isolates (data not shown). The ML tree based on nucleotide sequences at this locus (Fig. 2.4) clearly distinguishes between known APV clades and subclades. All 8 SA isolates analysed in this study group in clade A (FWPV-like viruses) with strong bootstrap support (Fig. 2.4). The isolates RP2 and FeP2 group in subclade A2 with a previously analysed SA APV, PEPV [20] and share 100%

nucleotide identity with the rest of the subclade (Fig. 2.4). Pi5 has a single nucleotide mutation and branches off from this subclade (Fig. 2.4). SP1, RP1 and LD1 share 100% nucleotide identity, grouping together with an isolate from a South Korean Oriental Turtle Dove [23] and a Spanish Great Bustard [23], in a new branch of subclade A3, annotated here as subclade A3.1 (Fig. 2.4). FeP1 and LD2 both exhibit one synonymous mutation in these sequences and group in subclade A3.1 a (Fig. 2.4).

H3L (VACV H3L; fpv140 locus).

Amplification of this region produced positive results of 1100bp for all 8 SA viruses (data not shown). Upon sequencing, these products were trimmed to 718bp and aligned to the available published APV sequences at this locus. The ML tree based on the nucleotide sequence of H3L (fpv 140) locus (Fig. 2.5) groups RP2, Pi5 and FeP2 in subclade A3 (A3c) which differs from the grouping in the P4b ML tree. Analysis of H3L provides greater resolution of clade A viruses. According to phylogenetic analysis of the P4b gene locus these viruses group in subclade A2 with previously analysed SA isolate, PEPV [20]. The viruses RP1, FeP1, SP1, LD1 and LD2 also group in subclade A3 (A3b) and share 98.75% nucleotide identity with RP2, Pi5 and FeP2.

VLTF-1 (VACV G8R; fpv126 locus).

All 8 SA APV isolates produced the expected 700bp product upon PCR amplification. These products were sequenced in duplicate and truncated to 570bp for alignment with published VLTF-1 orthologues. The ML tree was constructed using the Tamura 3-parameter model [251] with gamma distribution and the rate variation model allowed for some sites to be evolutionarily invariable ([+I], 28.7650% sites). The ML tree based on the VLTF-1 nucleotide sequence alignment (Fig. 2.6) shows that SA isolates belong to the APV genus and group with FWPV, in a separate clade from CNPV. Similar to the H3L gene tree (Fig. 2.5), analysis of the VLTF-1 locus indicates that FeP1, LD1, LD2, RP1 and SP1 group together within subclade A3 (A3b) (Fig. 2.6) with 100% nucleotide identity and FeP2, RP2 and Pi5 also group together with 100% nucleotide identity within subclade A3 (A3c).

fpv 175-176 (VACV A11R-A12R).

PCR of this region produced the expected 700bp product for all 8 analysed isolates. The tree based on the nucleotide sequences of this conserved region (Fig. 2.7) provided even further resolution of subclade A3c, grouping the viruses Pi5 and FeP2

(A3c (2)) separately from RP2 (A3c (1)) with strong bootstrap support. At the VLTF locus, these 3 viruses shared 100% nucleotide identity, and in the P4b gene Pi5 had a single base pair difference.

2.4 Discussion

This study compares the gross pathological and histopathological characteristics of CAMs infected with 9 different APV isolates. Studies describing gross pathology and histology of different APVs in CAMs have been done elsewhere, including Italy [19] and Egypt [233]. This is the first comparison of the growth characteristics of different APVs isolated from different bird species in South Africa. Different APV were grown using the same method and each virus stock was titrated so that a constant amount of virus was inoculated onto each CAM. This allowed for accurate comparisons of growth characteristics between viruses isolated from different bird species and geographical regions.

Manarolla et al. (2010) reported variable levels of thickening, ranging from mild to severe, in CAMs infected with APV isolates from Italy [19]. In an Egyptian study, isolates from chickens and a turkey produced compact, grayish-white pocks and marked thickening of the infected CAM tissue [233]. In this same study, a PGPV isolate produced nodular yellowish pocks and moderate thickening of the CAM tissue [233]. SA APV isolates also exhibited differing pock morphologies and degrees of membrane thickening (Tables 2.2 and 2.3).

Interestingly, all viruses isolated from pigeons (RP2, Pi5, FeP2) produced white pocks of variable size except for those isolates where the membrane thickening was so severe that no individual pocks were visible (FeP1, RP1). At lower titres (10^2, 10^1 PFU/egg) where membrane thickening was reduced, these viruses produced distinct white pocks (not shown). This pock morphology in SA pigeonpox virus isolates was different from the yellowish nodular pocks seen in CAMs infected with an Egyptian pigeonpox virus isolate [233].

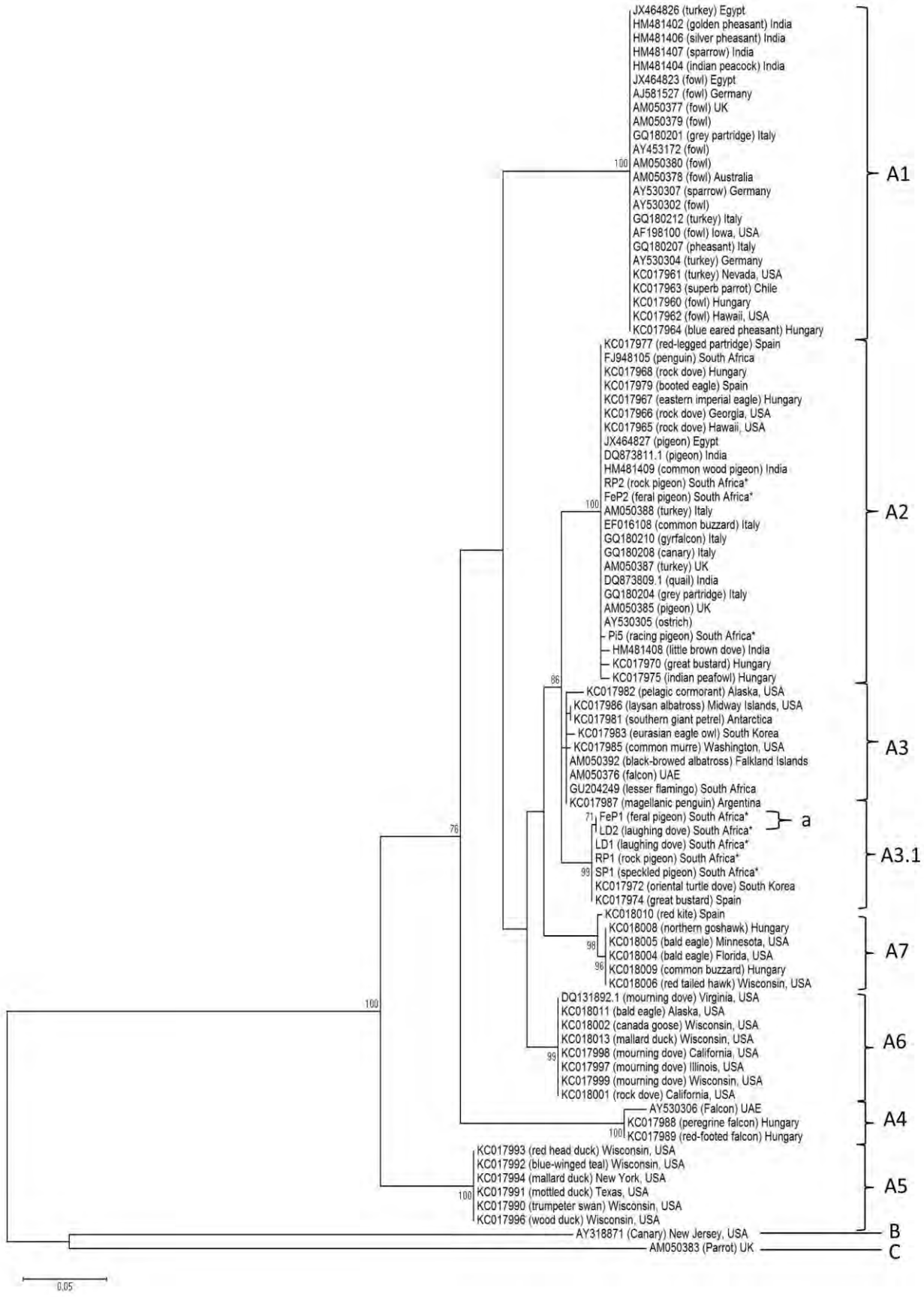


Figure 2.4 Maximum Likelihood tree based on the MUSCLE nucleotide alignments of P4b (fpv 167, VACV A3L). Novel South African isolates (SP1, RP2, LD2, Pi5, FeP2, LD1, FeP1 and RP1) (marked with asterisks; see Table 1 for abbreviations) were aligned to published sequences from Genbank (See annotated accession numbers). This ML tree was constructed using the Tamura 3-parameter model with gamma distrubution, and a bootstrap test of 100 replicate samples.

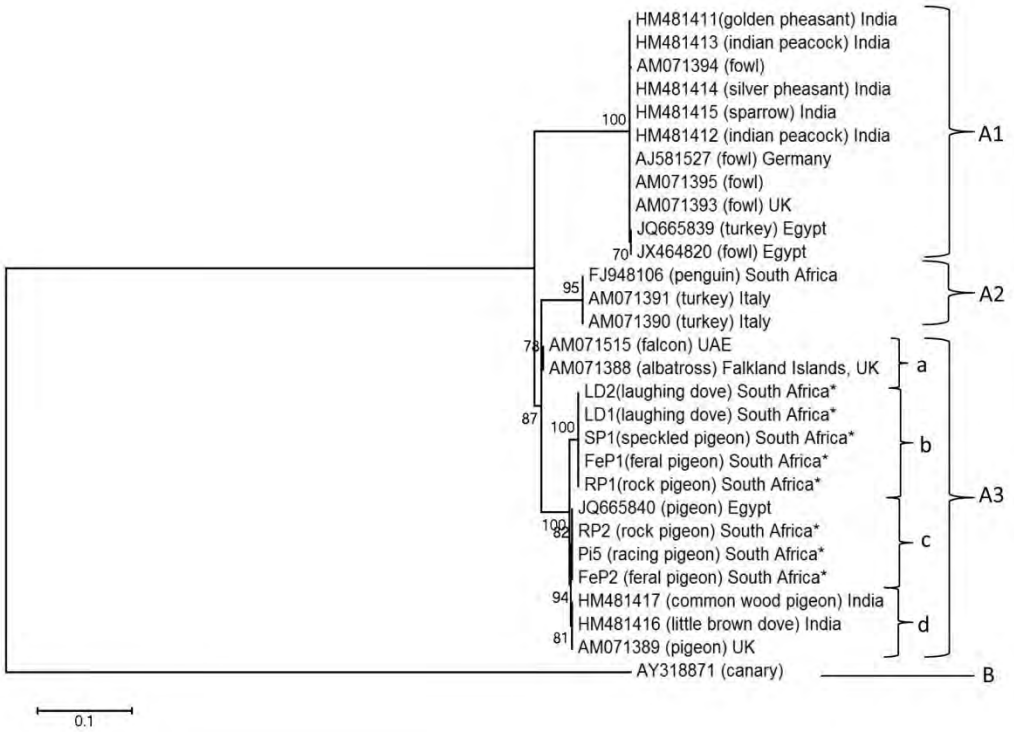


Figure 2.5 Maximum Likelihood tree based on the MUSCLE nucleotide alignment of the region corresponding to the p35 gene locus (*fpv*140, VACV H3L). Novel South African isolates (SP1, RP2, LD2, Pi5, FeP2, LD1, FeP1 and RP1) (marked with asterisks; see Table 1 for abbreviations) were aligned to published sequences from Genbank (See annotated accession numbers). This ML tree was constructed using the Tamura 3-parameter model with gamma distrubution, and a bootstrap test of 100 replicate samples.

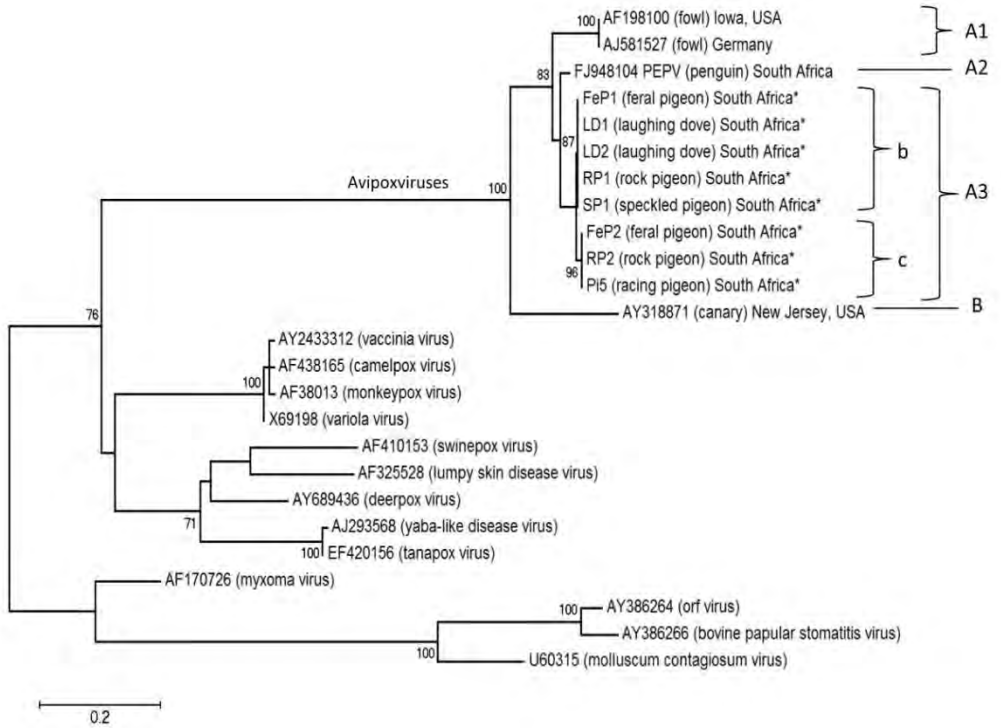


Figure 2.6 Maximum Likelihood trees based on the MUSCLE nucleotide alignment of the VLTF-1 gene (fpv126, VACV G8R). Novel South African isolates (SP1, RP2, LD2, Pi5, FeP2, LD1, FeP1 and RP1) (marked with asterisks; see Table 1 for abbreviations) were aligned to published sequences from Genbank (See annotated accession numbers). This ML tree was constructed using the Tamura 3-parameter model model with gamma distrubution, and a bootstrap test of 100 replicate samples.

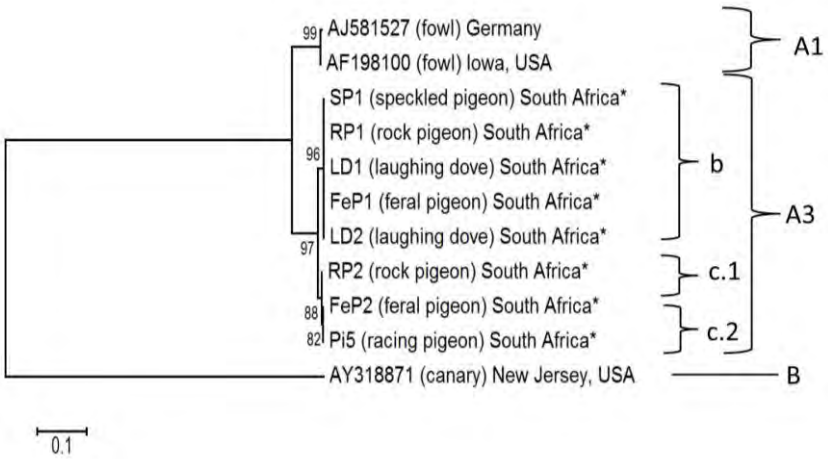


Figure 2.7 Maximum Likelihood tree based on the MUSCLE nucleotide alignment of the region corresponding to the locus, *fpv* 175-176 (VACV A11R-A12L). Novel South African isolates (SP1, RP2, LD2, Pi5, FeP2, LD1, FeP1 and RP1) (marked with asterisks; see Table 1 for abbreviations) were aligned to published sequences from Genbank (See annotated accession numbers). This ML tree was constructed using the Tamura 3-parameter mode with gamma distrubution, and a bootstrap test of 100 replicate samples.

While few studies have compared APV growth characteristics on CAMs, there have been many reports which describe differences in growth characteristics of the OPV [228-232]. Factors that influence poxvirus growth on CAMs include incubation temperature [232], age of embryos and the source of eggs [253]. Variability in pock colour has also been ascribed to mutation of specific viral genes [228, 229].

The FWPV vaccine strain (DCEP 25 modified strain (Merial)) produced red pocks with signs of haemorrhage (Fig. 2.3). Histology revealed that the FWPV-infected membranes showed signs of extensive angiogenesis and large blood vessels full of red blood cells. This is not dissimilar to the red pock phenotype induced by CPV. Unlike the pock phenotype of most other OPV, wild type CPV produces hemorrhagic red pocks on CAMs. However, CPV can produce spontaneous white-pock variants resulting from the deletion or mutation of a specific gene encoding the serine proteinase inhibitor (serpin), CrmA (SPI-2) protein [228, 229]. On histological examination, the CPV red pock is shown to lack inflammatory cells and have increased virus antigen and infectivity levels [238]. The CPV white pock phenotype is characterised by the presence of large numbers of heterophils and macrophages [231, 238] and produces extensive thickening of CAM tissue caused by proliferation of the epidermal and mesodermal cells [254]. Therefore, different phenotypes or growth characteristics may be indicative of different levels of immune responses in the CAM tissue [231, 238], caused by genetic make-up of the virus. It is unknown why this vaccine strain of FWPV produces such haemorrhagic pocks compared to

the other APV isolates. FWPV encodes 5 serpin homologues (*fpv*010, *fpv*040, *fpv*044, *fpv*204 and *fpv*251) and 2 homologues of cellular β-nerve growth factor (β-NGF) (*fpv*072, *fpv*076), which, when expressed by the virus, may interfere with early innate immune responses and may be important for viral infection [25]. It could be speculated that one or more of these serpin genes may be responsible for the red pock phenotype observed in FWPV-infected CAMs.

Several genes over and above the serpins have been associated with differences in phenotype of different poxviruses. VACV C22L codes for a tumor necrosis factor receptor (TNF-R) homologue which inhibits inflammation [255, 256]. FWPV encodes a gene similar to IL-18-binding protein (*fpv*073), which may inhibit inflammation [25]. It is possible that the viruses which do not cause significant inflammation (CAM thickening), such as CNPV (Canary), LD2 (Laughing Dove), Pi5 (Racing Pigeon) and RP2 (Rock Pigeon), may contain one or more of these anti-inflammatory genes or novel anti-inflammatory genes which may be responsible for their phenotype on CAMs. Whole genome sequencing and gene function analysis will be necessary to determine the cause of the different growth phenotypes of these viruses.

Differences in virus-induced responses in the CAMs, such as membrane thickening, immune cell infiltration, angiogenesis and hyperplasia, were observed in this study and one can only speculate why these differences exist. In the CAM model, administration of transforming growth factor β1 (TGF-β1) initiates a response that is similar in appearance to the CAM tissue infected by the isolates that caused extensive inflammation, namely FeP1 (Feral Pigeon), RP1 (Rock Pigeon), FeP2 (Feral Pigeon) and LD1 (Laughing Dove) [257].

TGF-β1 has proinflammatory properties and can inhibit growth, increase cellular accumulation through chemotaxis or cellular migration and increase microvascular angiogenesis. It is important in wound healing, tumour progression and embryogenesis [257, 258]. The isolates mentioned above caused epithelial and mesodermal thickening due to cellular hypertrophy and hyperplasia, angiogenesis, sloughing and infiltration of mononuclear immune cells which is similar to the appearance of CAM tissue that has been treated with TGF-β1 [257].

FWPV (*fpv*080) encodes a homologue of the eukaryotic TGF-β which is thought to be involved in suppression of the host immune response and/or cell growth and

differentation [25]. It is possible that the viruses that cause inflammation (FeP1, RP1) could encode functional homologues of a TGF-like protein.

The proliferative diseases caused by several poxviruses, including MCV have been attributed to the production of epidermal growth factor – like (EGF-like) proteins by virally infected cells [259, 260]. Poxvirus EGF-like growth factors have been shown to stimulate cell proliferation at regions of viral replication [261]. FWPV (*fpv*211) also encodes an EGF-like domain [25] which may contribute to the hyperplasia observed in FWPV-infected tissue [262]. FWPV does not produce extensive membrane thickening; however, hyperplasia is observed when compared to uninfected CAM tissue. The viruses causing inflammation (FeP1, RP1, FeP2, LD1), may contain additional growth factor-like genes, which may cause the increased inflammation observed in the CAMs infected with these viruses.

Although the phylogenetic analysis of 4 conserved regions suggests that these viruses are closely related to one another, the variation in pock morphology and histology amongst these viruses indicates that many of our novel APVs differ significantly. For example, phenotypically, Pi5 (Racing Pigeon) and FeP2 (Feral Pigeon) differ considerably, with FeP2 causing more hyperplasia and membrane thickening than Pi5. Phylogenetically, however, they group together in subclade A3c (according to the VLTF-1, H3L and fpv175-176 loci). In addition, the isolates LD2 (Laughing Dove) and FeP1 (Feral pigeon), which were both obtained from the same geographical region (Port Elizabeth (PE)) differ with regard to their pock morphology and histology but cluster together in the subclade A3b. The differences in resolution between the trees constructed based on these four loci, suggest that the viruses may vary at different genomic regions, and that phylogenetic analysis based on a few highly conserved genes may not be sufficient to determine divergence amongst the APVs. This has also been observed in the two different FWPV strains that have been sequenced. FPVUS and FP9 both group in clade A2, and yet analysis of the genome sequences of FP9 and FPVUS revealed 118 mutations, affecting 71 ORFs [27]. More detailed analyses, in the form of genomic sequencing, microarray analysis to reveal dysregulation of host immunomodulatory proteins and pathway analysis, will help to explain why these differences exist.

It is important to note that the viruses in this study were isolated from discrete geographical locations, up to nearly 1000kms apart (Cape Town (CT) to Port Elizabeth (PE) - 790kms). This geographical separation does not, however, coincide

with clustering of the viruses according to the phylogenetic trees, with isolates from the same region grouping separately. Although FeP1 and LD2, both from PE, group together in subclade A3b, FeP2, from the same region in PE, groups in subclade A3c (according to the ML trees constructed based on the H3L, VLTF-1 and fpv175-176 loci). All three viruses from PE differ with respect to CAM morphology. Moreover, several viruses from different regions cluster together phylogenetically. This is seen in RP1, isolated from a Rock Pigeon in Table View, CT, which clusters together in subclade A3b with FeP1 (Feral Pigeon) and LD2 (Laughing Dove) isolated in PE. Pi5 (Racing Pigeon) and FeP2 (Feral Pigeon) also cluster together in subclade A3c, and were isolated from Grabouw in the Western Cape, and PE in the Eastern Cape respectively. The A3b and A3c viruses differ with respect to pock and CAM morphology.

In a similar study conducted in New Zealand (NZ), where APV infection is known to be endemic in free-ranging bird populations, it was shown that most NZ APV isolates, including those isolated from a Song Thrush (*Turdus philomelos*), Saddlebacks (*Philesturnus carunculatus rufusater*) (*Philesturnus carunculatus carunculatus*), Sparrow (*Passer domesticus*), Black Robin (*Petroica traversi*), Silvereye (*Zosterops lateralis*), Shore Plovers (*Thinornis novaeseelandiae*), Variable Oyster Catchers (*Haematopus unicolor*) and a Paradise Shelduck (*Tadorna variegate*), belong to subclade A1, sharing 100% nucleotide identity to the FWPV vaccine strain used in NZ [263]. This suggests that several NZ free-ranging birds are susceptible to the specific A1 strain used as an attenuated fowlpox vaccine. Certain NZ samples grouped in subclades A3 and B1 [263]. APVs isolated from SA birds all group within Clade A (FWPV-like viruses), in either subclades A2 or A3. Although we know that FWPV exists in A poultry, none of the viruses analysed in our study were identical to SA FWPV or FWPV vaccine strains used in SA (Clade A1) (data not shown).

Based on the phylogenetic analysis of four conserved regions, the viruses characterised from SA columbiformes cluster into two groups. The viruses from Feral Pigeon (FeP2), Rock Pigeon (RP2) and Racing Pigeon (Pi5) group in subclade A3c and the viruses from a Rock Pigeon (RP1), two from Laughing Doves (LD1, LD2), a Feral Pigeon (FeP1), and a Juvenile Rock Pigeon (SP1) group in subclade A3b. Therefore, in this study as well as others [18, 19, 263] APVs from the same species of bird are classified in different subclades. Conversely, it has also been shown that the same viruses can infect different birds [233, 244, 264].

Pigeonpox viruses (PGPVTP2, PGPVP, HM481409, HM481408) group in subclade A2 according to P4b [18, 244, 265] and based on the H3L gene, they group in subclade A3, along with isolates from an albatross, falcon and flamingo [18, 233, 244]. Pigeonpox isolates grouping in subclades B1 and B2 [18, 19, 245] have also been noted.

The complicated nature of the host-range of APVs has led to the suggestion that the taxonomy of these viruses should be changed. Jarmin et al. (2006) criticised the host species-based approach to APV taxonomy because different viral samples taken from a particular species can be found to group in different subclades or clades [18]. This was seen in our study where isolates from Feral Pigeons, FeP1 and FeP2 grouped separately (subclade A3b and A3c respectively) and also differed considerably with regard to their growth characteristics. Similarly, this was also seen in viruses isolated from 2 Rock Pigeons, RP1 and RP2. Therefore, the results of this study, along with several others [18, 19, 233, 244], provide evidence that the existing host species-based classification may be oversimplified for the complicated host-range of APVs.

Preliminary phylogenetic analysis and characterisation of the pathology of novel SA APVs on CAMs has been performed in this study. For the first time, information is available on which APVs are circulating in SA birds. According to the phylogenetic analyses presented here, the viruses circulating in SA birds group with FWPV-like viruses in clade A, subclades A2 and A3, and are shown to cluster into two groups which are seemingly independent of the species of bird from which they were isolated. Current convention is to name the virus after the species in which it was originally described; however, it is suggested that alterations to the existing taxonomy of APV be made, which take into account genetic diversity and the variability of virus-host interactions, growth characteristics and infectivity. Prior to this study, the genomes of only three APVs had been published; a pathogenic United States strain of fowlpox (FPVUS) [25], a plaque purified, tissue culture adapted, attenuated European strain of FWPV (FP9) [27] and a virulent CNPV (CNPVATCC VR-111) isolate [24]. FPVUS and FP9 group in clade A1 and CNPVATTC groups in clade B1. According to the genetic regions fpv 26 (VLTF-1), fpv167 (P4b), fpv140 (H3l) and fpv175-176 (VACV A11R-A12L), the novel APVs analysed in this study are grouped differently from the three APVs (FPVUS, FP9 and CNPVATCC) which have been sequenced [24, 25, 27].

As discussed in chapter 1, section 1.3.5, APVs make promising candidates for use as vaccine vectors. Considerable success has been demonstrated using the canarypoxvirus ALVAC as a vaccine vector [47, 60, 75, 76]. However there is room for improvement of the APV vaccine vectors and it is possible that novel APVs may make better vaccine vectors than the established ones being used, namely FWPV and CNPV, for specific diseases. Therefore, there is a need for the discovery of novel vectors and the development of improved vectors. In this study we show that although the phylogenetic analysis of highly conserved genetic regions indicates that the SA APVs are closely related to each other, variation in growth characteristics on CAMs suggests that many of our novel APVs differ significantly. From these results we have chosen one novel APV, FeP2, for further characterisation and determination of its potential for use as a vaccine vector candidate. This virus was chosen as a result of its interesting phenotype on CAMs and the fact that, phylogenetically, it grouped differently according to the different genetic regions analysed. Furthermore, this was the only one of our SA viruses which was able to grow in tissue culture (data not presented for all viruses, see section 3.3.1. for FeP2) Further characterisation in the form of growth kinetics, transmission electron microscopy and the induction or inhibition of apoptosis by this virus in different cell lines would provide valuable information regarding the *in vitro* behaviour of FeP2. More detailed analyses, in the form of genomic sequencing as well as pathway analysis/immunomodulation by microarray, will allow for more thorough differentiation of APVs.

Chapter 3

Analysis of the growth and induction/inhibition of apoptosis of a South African pigeonpox virus (FeP2) in permissive and non-permissive cell lines and comparison to other poxviruses

3.1 Introduction

In comparison to the extensively studied Orthopoxvirus (OPV) genus, little is known about the morphogenesis and growth characteristics of APVs. APVs have a restricted host-range and are only able to productively replicate in permissive avian species. However, APVs can infect non-avian hosts and are generally accepted to undergo abortive replication in mammalian cells [45, 46, 71]. Limited exceptions have been reported and replication of APVs in some mammalian cells, such as embryonic bovine tracheal cells [266] and baby hamster kidney cells have been reported [267].

APVs are commonly grown in primary chick embryo fibroblasts (CEFs). However growth in other cell lines, such as the Japanese quail cell line, QT-35 [268, 269] has been reported, although the presence of endogenous herpes virus and Marek‘s disease virus (MDV) in these cells limits their use for vaccine preparation [270]. CEF cells have been shown to be permissive for CNPV and FWPV [271]. Penguinpox virus (PEPV) does not produce infectious progeny in CEF cells, although progeny virions were apparently morphologically complete following one cycle of infection [71]. No permissive avian cell line for PEPV has been identified [71].

In permissive cells, APVs follow a similar process of morphogenesis as other poxviruses (described in detail in section 1.1.3) [272, 273]. However, in contrast to the OPVs which exit the infected cell via exocytosis of the IEV [274], APV MV preferentially bud out of membranes. Exocytosis can also occur [273]. In non-permissive cells there is a block in the APV life cycle. For FWPV and PEPV this block occurs after the formation of IV [71, 275]; but for CNPV the block occurs prior to viral DNA replication [45].

FWPV is capable of infection of mammalian cells and expression of both early and late genes in fibroblast-like monkey cells (Vero, CV-1) and human MRC-5 cells. In these cells FWPV undergoes DNA replication and morphogenesis proceeds to the formation of immature particles, similar to that observed for MVA in human cells [275]. Notably, in the epithelial-like HeLa human cell line, barely any FWPV gene expression was observed [275]. Preliminary experiments on CNPV growth in Vero cells indicate that, in contrast to FWPV, CNPV-infection fails to produce any detectable expression of post-replicative genes [275]. Pacchioni et al (2010) also found that human cells are less permissive to APV replication compared to monkey

cells and that FWPV advances further than CNPV in its life cycle in monkey cells [271]. Virus-like particles were observed following infection of replication restrictive human cells with a FWPV recombinant containing a HIV *gag*/*pol* transgene, under the control of the H6 early/late promoter, demonstrating that FWPV infection and transgene expression can occur in the absence of visible viral morphogenesis [271].

Few direct comparisons of poxvirus vector candidates have been reported. In a comparison of MVA and CNPV, antigen production was shown to be greater (due to longer duration of antigen production) by recombinant MVA than recombinant CNPV. Increased levels of apoptosis induction in non-permissive HeLa cells were observed in response to CNPV compared to MVA; however, inclusion of a *gag*/*pro*/*env* expression cassette altered apoptosis kinetics such that both recombinant viruses induced similar levels of apoptosis. These results suggest that differences in both poxvirus vector- as well as insert-host interactions are relevant to the use of poxviruses as vaccine vectors [136].

Significant differences in vector-generated cytopathic effect (CPE), growth characteristics, apoptosis induction and stage of replication block were observed in cell culture systems in a comparison between the closely related VACV derivatives, MVA and NYVAC [276]. The growth characteristics of MVA and NYVAC under permissive and non-permissive conditions were assessed in BHK-21 and HeLa cells respectively [276]. Under permissive conditions (BHK-21 cells), MVA and NYVAC growth kinetics were similar. In HeLa cells, MVA replication is blocked following formation of IVs without alteration of early or late gene expression; however, in NYVAC-infected HeLa cells, the block in morphogenesis occurs prior to IV formation [276, 277]. Transmission electron microscopy indicated that upon infection with NYVAC, and not MVA, HeLa cells display morphological and biochemical features of apoptotic cell death, including chromatin condensation, cytoplasmic vacuolisation and DNA fragmentation [276].

A study of the morphogenesis of novel APVs in permissive and non-permissive mammalian cell lines by means of transmission electron microscopy will reveal how far the virus progresses in its life-cycle. In non-permissive host cells, different poxviruses can progress to different stages of morphogenesis [71, 271, 276, 277]. The stage at which the morphogenesis of the virus is blocked in non-permissive cells may impact on the expression of transgenes and therefore may have an impact on immunogenicity of the vaccines. A longer period of transgene expression and the

induction of local inflammation by the vaccine vector may result in the improved recruitment of antigen-presenting cells at the site of inoculation and possibly, the generation of a better immune response [271].

Virus induction or inhibition of apoptosis is an important consideration when determining the potential of a poxvirus for use as a vaccine vector. Apoptosis, or programmed cell death, eliminates virally infected, damaged or unwanted cells without disruption of surrounding tissues (reviewed in chapter 1, section 1.4.3.1). Poxviruses have evolved several mechanisms which modulate virus infection-induced apoptosis in the host [278, 279] (reviewed in chapter 1, section 1.4.4). This has important implications in vaccine design because if apoptosis occurs too early in the life cycle of the virus, gene expression and antigen presentation will be affected. Apoptosis is, however, desirable once the transgene of a recombinant vaccine has been expressed, as the formation of apoptotic bodies loaded with antigen and the uptake thereof via the alternative class I antigen presentation pathway (cross-presentation) is important in the induction of an immune response [133]. The mechanisms of apoptosis and its effect on vaccine efficacy are not clearly understood. Both induction and delay/prevention of apoptosis by poxvirus vectors have been shown to improve foreign antigen expression as well as immunogenicity [134-136]. It would be important to assess any novel vaccine vector candidates for the ability to induce or inhibit apoptosis in permissive and non-permissive cells. This is a potential avenue for the improvement of APV vectors, with the insertion or deletion of immunomodulatory genes.

In this study, a novel South African APV isolated from a Feral Pigeon (*Columba livia*) (FeP2) has been characterised in terms of growth, morphogenesis and induction/inhibition of apoptosis in permissive and non-permissive cells. This study will provide fundamental characterisation and a better understanding of *in vitro* behaviour of this poxvirus.

3.2 Materials and methods

3.2.1 Virus isolates

Poxviruses were obtained from various sources (Table 3.1) and grown as described in chapter 2, section 2.2.1. PEPV seedstock was kindly obtained from Olivia Carulei a fellow PhD student at University of Cape Town, originally isolated by D. Kow and

K. Dumbell [71]. MVA was obtained from Prof. K. Dumbell's collection housed at the University of Cape Town, originally obtained from Prof. A. Mayr (Veterinary Faculty, University of Munich, Munich, Germany). CNPV and FWPV are described in chapter 2, section 2.2.1.

Table 3.1. List of avipoxvirus isolates used for further analysis.

Virus Abbreviation	Host Species	Source
FeP1 (PGPV11K FP1)	Feral pigeon *Columba livia*	Dr Peter Kroon: Southern Cross Veterinary Clinic, Port Elizabeth
PEPV (PEPV San92)	Penguin *Spheniscus demersus*	SANCCOB: Southern African Foundation for the Conservation of Coastal Birds, Cape Town
FWPV Fowl pox virus, (DCEP 25 modified strain (Merial))	Fowl *Gallus gallus*	Merial, US
CNPV (wild type)	Canary *Serinus canaria*	From Dumbell collection, originally from Mayr
Modified vaccinia Ankara (MVA)	unknown	From Dumbell collection, originally from Mayr

3.2.2 Cell lines and culture conditions

Human cervix adenocarcinoma cells (HeLa, ATCC CCL-2™), Syrian baby hamster kidney cells (BHK-21, ATCC CCL-10™) and Japanese quail myoblasts (QM7, CRL-1962™) were obtained from and grown under conditions suggested by the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained at 37°C in 5% CO_2. HeLa and BHK-21 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS) (Biochrom, Cambridge, UK), 1% penicillin and streptomycin (Lonza, Basel, Switzerland) and 0.001mg/ml Fungin (Invivogen, San Diego, USA). QM7 cells were grown in 80% Medium 199 with Earle's Balanced Salt Solution (Lonza, Basel, Switzerland), 10% tryptose phosphate broth, 10% FCS, 1% penicillin and streptomycin and 0.001mg/ml Fungin (Invivogen, San Diego, CA, USA).

Fresh primary CEFs were isolated from 10-11 day old embryonated SPF White Leghorn chicken eggs which were obtained from Avifarms (Pty) Ltd (Lyttelton, South Africa). Embryos were aseptically removed from the egg, decapitated and the limbs carefully removed. They were then washed twice in PBS and incubated with 0.2% trypsin (Lonza, Basel, Switzerland) in PBS at 37°C for 30-60mins. An equal volume of FCS was added to neutralize the trypsin and the suspension was filtered through

autoclaved cheesecloth. The filtrate was centrifuged at 1500rpm (Eppendorf 5810) and the cell pellet was resuspended in DMEM with 10% FCS and counted. Cells were seeded into 75cm^2 polystyrene tissue culture flasks (Corning®, New York, NY, USA) at 2 x 10^6 cells per flask and grown in DMEM supplemented with 10% FCS (Biochrom, Cambridge, UK), 1% penicillin and streptomycin (Lonza, Basel, Switzerland) and 0.001mg/ml Fungin (Invivogen, San Diego,CA, USA).

3.2.3 Virus growth in different cell lines

Monolayers of HeLa, BHK-21, QM7 and CEFs in 25cm^2 flasks were grown to 80% confluency. Virus was diluted in DMEM, without serum but including 1% penicillin and streptomycin and 0.001mg/ml Fungin. The culture media was removed and cells were infected at a multiplicity of infection (m.o.i) of 0.01 PFU/cell. Following virus adsorption for 2 hours at 37°C, the inoculum was removed and replaced with fresh DMEM containing 4% FCS and incubated at 37°C with 5% CO_2. At different times post infection (0hrs, 24hrs, 48hrs, 72hrs, 96hrs and 120hrs), flasks were examined for CPE and placed at -20°C. Following four freeze/thaw cycles, the few attached cells remaining were scraped and the supernatants were collected. This was diluted and titrated on CAMs as described in chapter 2, section 2.2.2.

3.2.4 Electron microscopy

HeLa and CEF cells were seeded into 12 well plates and adhered cells were infected with FeP2 (moi=10) and allowed to adsorb for 1hr. Thereafter media containing 2% FCS was added and cells were incubated for 6hrs, 15hrs, 24hrs or 48hrs. The cells were then scraped and, together with the supernatant, were spun at 5000rpm for 5mins. The resulting pellet was then washed in 1ml PBS and subjected to centrifugation at 5000rpm for 5mins. The supernatant was then discarded and the pellet was resuspended in 100ul 2.5% glutaraldehyde and incubated overnight at 4°C. Following two washes in 0.1 M phosphate buffer pH 7.4, the cell pellets were resuspended in 4% low melting point agarose, allowed to set and cut into ~1mm^2 blocks. The blocks were then immersed in 0.5% tannic acid at room temperature for 1 hour and washed with PBS. Secondary fixation was performed in 1% (wt/vol) OsO_4 in 2xPBS for 1 hour at room temperature. Samples were then washed twice in PBS and twice in distilled water for 5 minutes for each wash. Samples were dehydrated through a 30-100% (vol/vol) ethanol series, infiltrated with acetone and

Spurrs resin (1:1, 3:1, 100%) (Polysciences Inc., Warrington, PA, USA) and embedded with Spurrs resin (100%). Polymerization of resin blocks was performed at 60°C for 24hrs. Ultra-thin sections were made using a diamond knife and stained with uranyl acetate and lead citrate. The specimens were observed under a Zeiss 912 OMEGA EFTEM or a FEI/Tecnai F20 CRYO FEGTEM transmission electron microscope (TEM) [71, 271, 276]. We acknowledge Mr Mohammed Jaffer and others at the UCT Electron Microscope Unit for training and assistance with the electron microscopy performed.

3.2.5 Measurement of induction or inhibition of apoptosis

Primary CEFs or HeLa cells were seeded in 10% DMEM at 2 x 10^4 cells per well in a 96 well plate. 24hrs later, the medium was removed and either 2% DMEM alone (uninfected control), 4ug Camptothecin alone (positive control), virus (moi=10) or virus and Camptothecin together (moi=10, 40ug/ml ie: 4ug) were added to the wells and incubated at 37°C for different times. Thereafter the enzyme-linked immunosorbant assay (ELISA)-based Cell Death Detection ELISAPLUS kit (Roche, Basel, Switzerland) protocol was performed as per the manufacturer‘s instructions (except that 2 x 10^4 cells per well were used instead of 1 x 10^4 cells per well in a 96 well plate). Plates were read at a wavelength of 405nm with a reference wavelength of 490nm**.**

3.3 Results

3.3.1 Growth kinetics of FeP2 in permissive and non-permissive cell lines and comparison to other poxviruses

Avian cells

If a virus is to be used as a potential vaccine vector, a permissive cell line is necessary for the construction of a recombinant virus as viral growth in tissue culture is needed for homologous recombination of the transfer vector with the APV genome. We therefore investigated the growth of FeP2 in two cell lines of avian origin, primary CEFs and Japanese quail myoblasts (QM7).

In primary CEFs, FeP2 showed an increase in virus titre of approximately 2 logs over 5 days (Fig. 3.1). Similarly, CNPV, FWPV, MVA also increased in virus titre from day 0 (0hrs) up to day 5 (120hrs), but not as much as FeP2 (Fig. 3.1). PEPV,

included in this experiment as a negative control, did not grow in primary CEFs and virus yield decreased over the 120hrs (Fig. 3.1) confirming previous studies [71]. Limited CPE in these cells was observed (not shown). MVA produced the highest viral yield, with an increase of more than 3 logs over 3 days (Fig. 3.1).

Growth of FeP2 in an additional, permanent cell line of avian origin (QM7) was investigated. Growth in QM7 cells was unproductive and virus titre decreased over 120hrs (Fig. 3.3). Infectious FeP2 appeared to be relatively stable in these cells and virus titre dropped less than 1 log fold (Fig. 3.3).

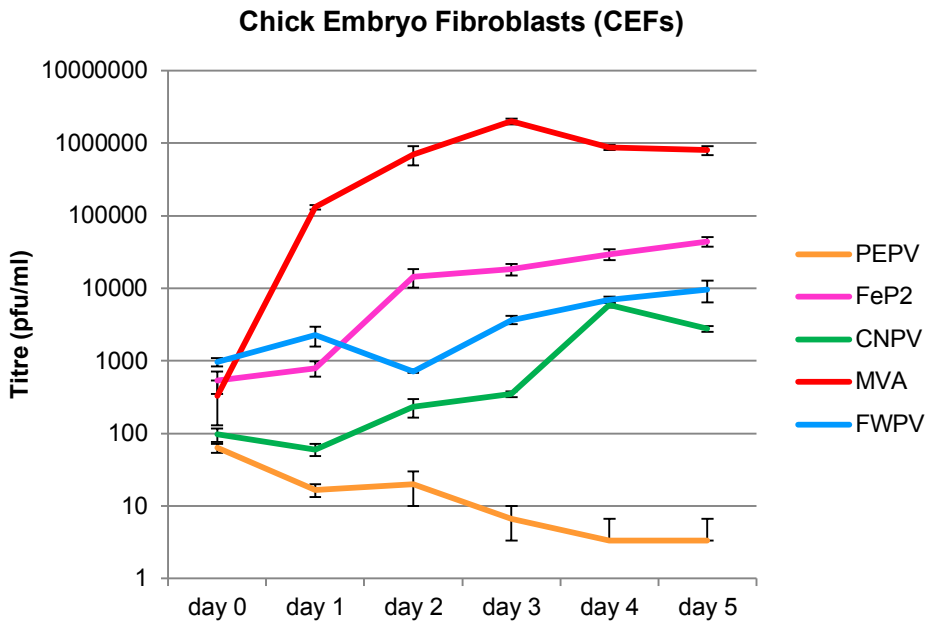


Figure 3.1. Virus growth of PEPV, FeP2, CNPV, MVA and FWPV in primary chick embryo fibroblast (CEF) cells. CEF cell monolayers were infected at 0.01 PFU/cell and harvested at different times post infection (day 0 (0hrs), day 1 (24hrs), day 2 (48hrs), day 3 (72hrs), day 4 (96hrs) and day 5 (120hrs)). Virus was titrated and averages of three titrations are shown with error bars calculated with standard error for each dataset.

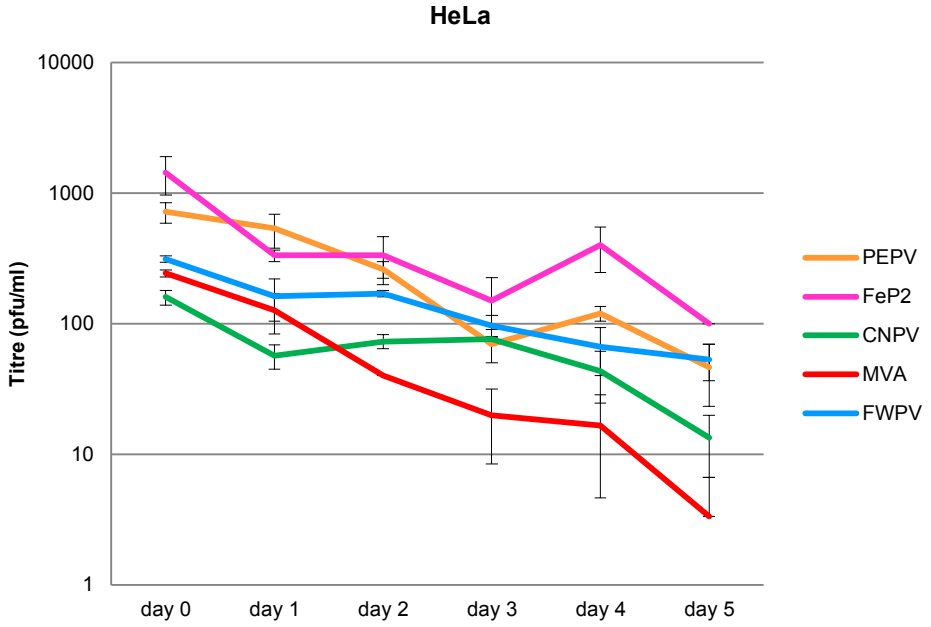


Figure 3.2. Virus growth of PEPV, FeP2, CNPV, MVA and FWPV in HeLa cells. HeLa cell monolayers were infected at 0.01 PFU/cell and harvested at different times post infection (day 0 (0hrs), day 1 (24hrs), day 2 (48hrs), day 3 (72hrs), day 4 (96hrs) and day 5 (120hrs)). Virus was titrated and averages of three titrations are shown with error bars calculated with standard error for each dataset.

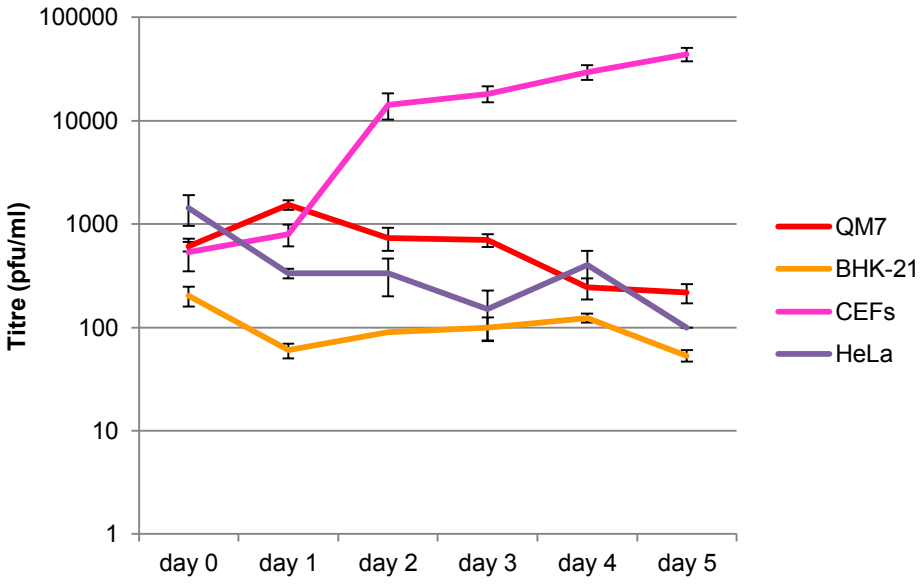


Figure 3.3. Virus growth of FeP2 in QM7, BHK-21, CEF and HeLa cells. Cell monolayers were infected at 0.01 PFU/cell. At different times post infection (day 0 (0hrs), day 1 (24hrs), day 2 (48hrs), day 3 (72hrs), day 4 (96hrs) and day 5 (120hrs)) virus was titrated and averages of three titrations are shown with error bars calculated with standard error for each dataset.

Mammalian cells

In order to establish whether or not FeP2 can multiply in mammalian cells, we investigated FeP2 growth kinetics over 120hrs in HeLa cells and compared this to the growth of CNPV, FWPV, PEPV and MVA. There was no increase in virus titre for any of the viruses and all viruses displayed similar patterns of growth kinetics (Fig.3.2).

Previous studies have shown that BHK-21 cells are permissive to certain APV strains [267]. We therefore investigated whether FeP2 could multiply in this cell line over 5 days (120hrs). Overall, no increase in virus titre was observed; however, stability of infectious FeP2 was demonstrated over 5 days (Fig. 3.3). In HeLa cells, there is more of a drop in FeP2 titre than in BHK-21 cells. This could mean that low levels of FeP2 replication is occurring.

3.3.2 Morphogenesis of FeP2

Permissive cells

To characterise the morphogenesis of FeP2 in permissive cells, transmission electron microscopy (TEM) was performed on infected CEFs at 15 and 24hrs post infection. In CEFs, morphogenesis of FeP2 followed the same sequence of events as those reported for PEPV [71], FWPV [271, 273] and CNPV [271]. Viral crescent membranes were seen surrounding cytoplasmic masses of granular material. These progressed to spherical IV with amorphous content (Fig. 3.4A-D). IV particles at various stages of development were observed (Fig. 3.4A-D). First partly open circular membranes originating from viral crescents enclose viral nucleoplasm to form closed double-shelled spheres consisting of homogenous granular matter (Fig. 3.4A-D). Thereafter, the contents become condensed and differentiate into the viral cores and lateral bodies and the virions become oblong MV particles (Fig. 3.4E, F, G and H). Mature particles become wrapped (enveloped) as they bud through the membranes of cytoplasmic organelles (possibly Golgi cisternae) (Fig. 3.4 E). No definite budding through plasma membranes was observed, however MV at different stages of maturity were observed in the extracellular space and cytoplasm of degenerating cells (Fig. 3.4F, G and H and not shown).

Non-permissive cells

In FeP2-infected HeLa cells, FeP2 particles were seen adhering to cell membranes or in the extracellular space (Fig. 3.5A); however no virions were seen entering any cells. Areas of electron dense viroplasm (v) were observed in some cells (Fig. 3.5B) and suspected aberrant forms of IV particles were present (Fig. 3.5C), however this cannot be confirmed. In one isolated cell (Fig. 3.5D) several mature virus particles, similar to those observed in permissive cells (Fig. 3.4) were observed within the cytoplasm, possibly a result of a clump of virus particles entering the cell. No evidence of productive infection or early morphogenesis was observed at 6hrs, 15hrs, 24hrs or 48hrs (not shown).

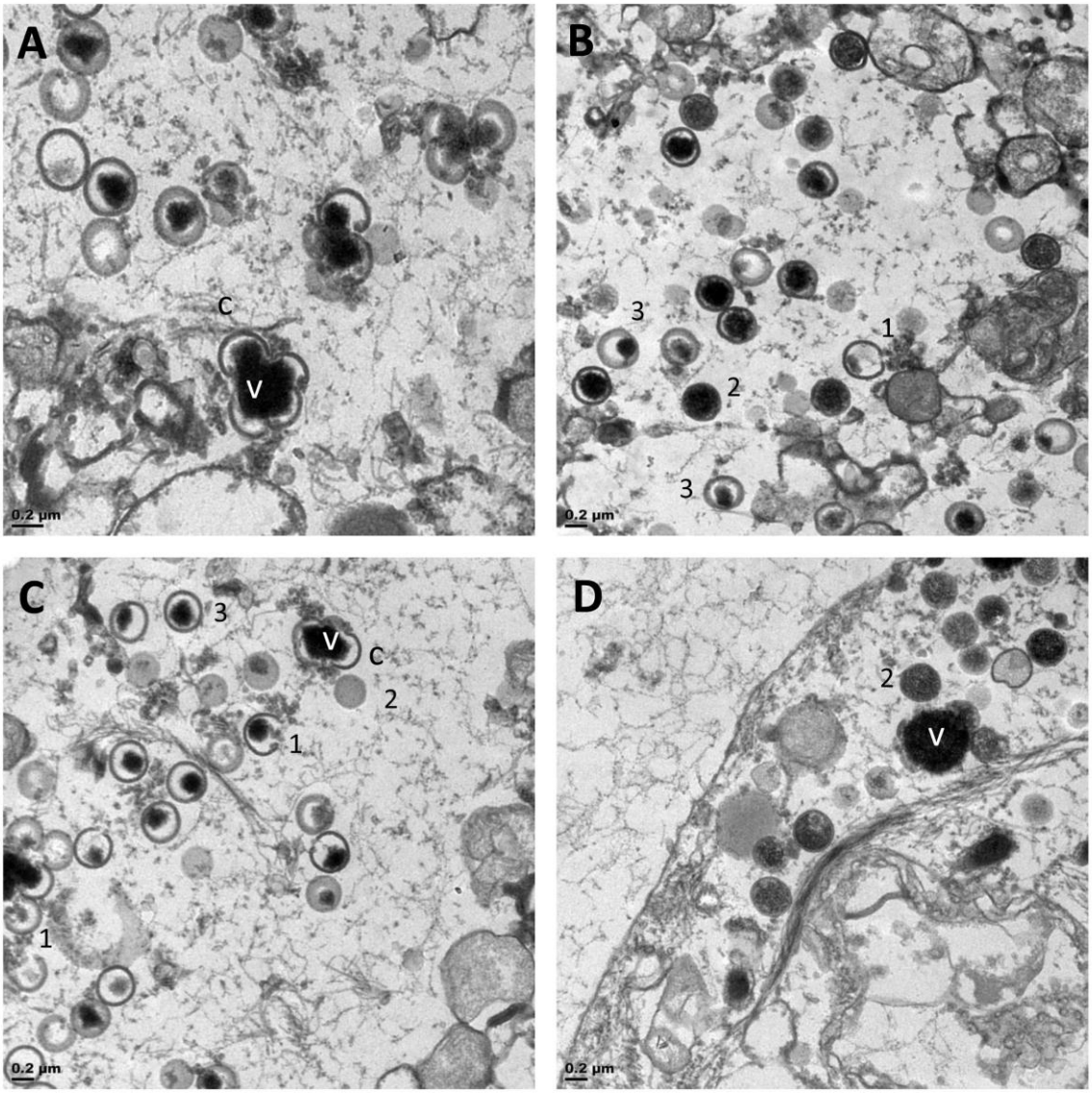


Figure 3.4 Morphogenesis of FeP2 in CEF cells. Electron microscopy was performed 15 and 24 hrs p.i**.** Pools of viroplasm (V) can be seen surrounded by viral crescent membranes (c). Immature virus (IV) particles at various stages of development are seen in the cytoplasm of the infected cell: (1) partly open circular membrane structures enclosing/associated with nucleoplasm. (2) Completely closed spherical particles with amorphous content. (3) IV contents condense and become differentiated.

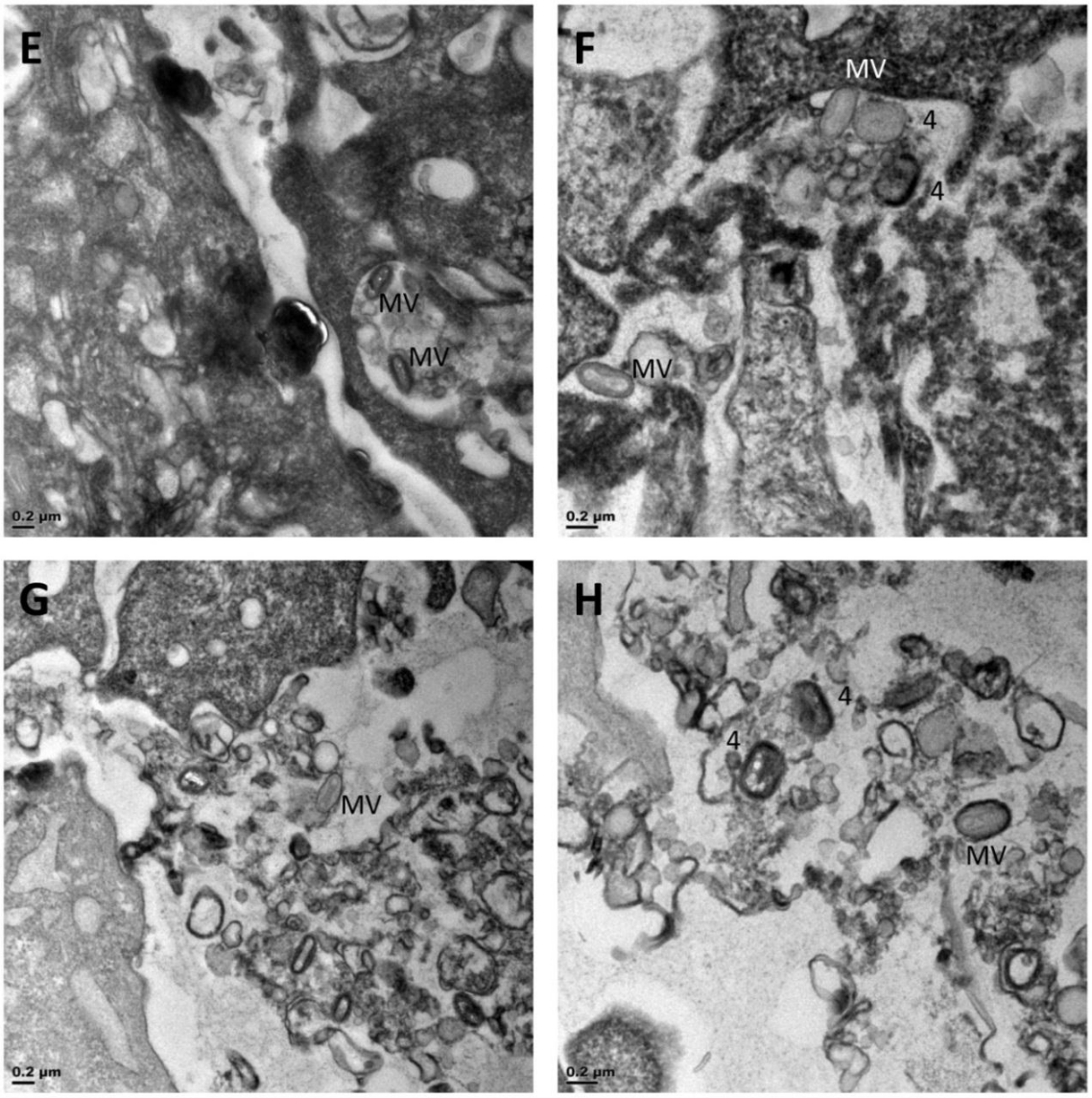


Figure 3.4 (Continued) Morphogenesis of FeP2 in CEF cells. (4) The contents of the IV contracts and become differentiated, forming oblong mature virus particles (MV) with a characteristic biconcave core. Mature particles become wrapped (enveloped) as they bud through the membranes of cytoplasmic organelles (possibly Golgi cisternae) (E). MV were observed in the extracellular space cytoplasm of degenerating cells (F, G and H and not shown)

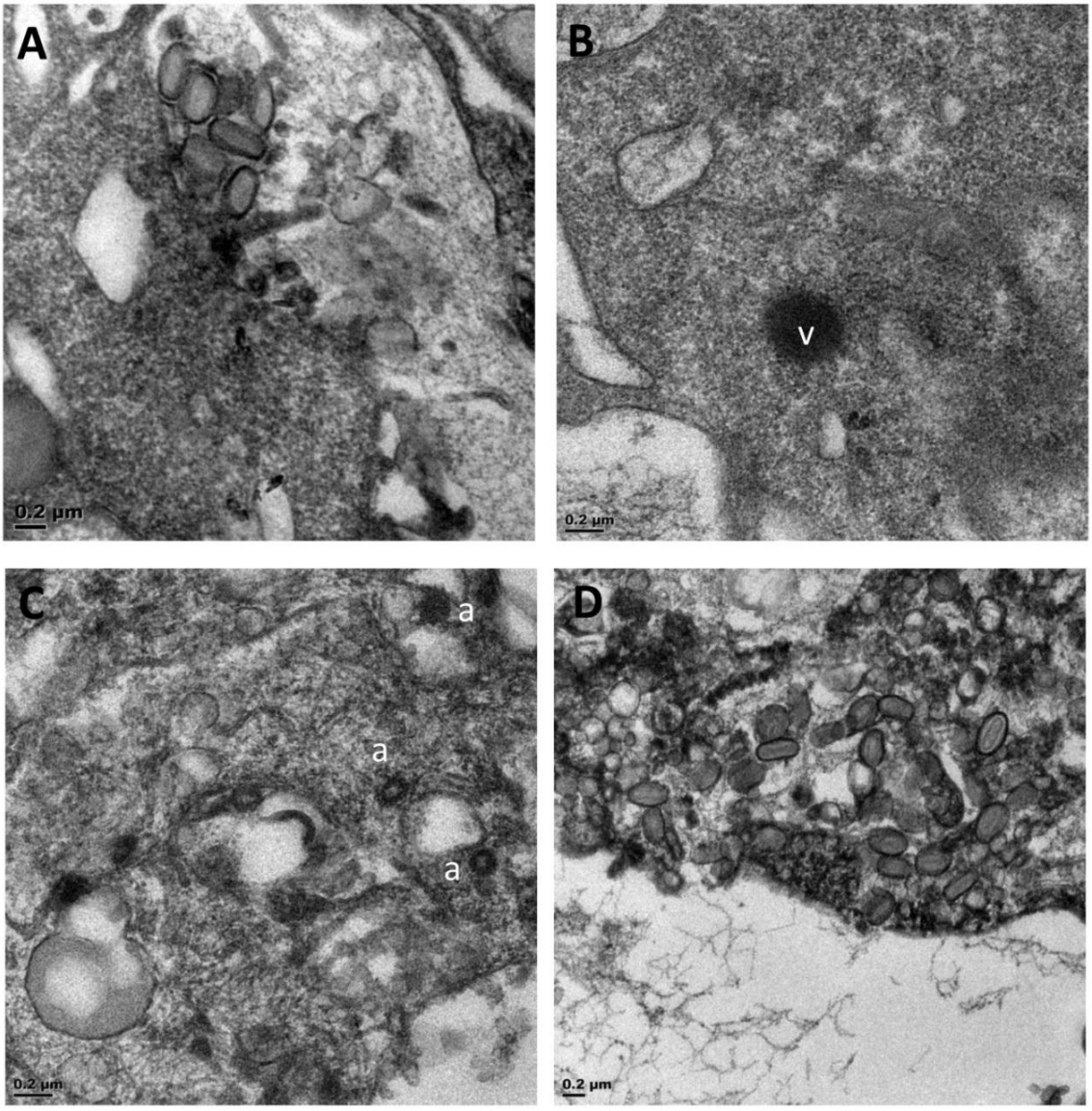


Figure 3.5 Electron microscopy of FeP2 in HeLa cells. FeP2 particles are seen adhering to cell membranes or in the extracellular space (A). (B) Areas of electron dense viroplasm (v) are observed in some cells and suspected aberrant forms of immature virus particles are present (a). In one isolated cell (D) several mature virus particles were observed within the cytoplasm.

3.3.3 Investigation of the induction/inhibition of apoptosis by FeP2 in comparison to different poxviruses in permissive and non-permissive cells

We performed a quantitative investigation of apoptosis induction/inhibition by FeP2 in comparison to CNPV, FWPV, PEPV and MVA using an ELISA-based assay (Roche Cell Death Detection ELISAPLUS) for detection of histone-complexed DNA fragments in the cytoplasm of permissive (primary CEFs) and non-permissive (HeLa) cells.

To examine induction of apoptosis, cells were infected with virus at a moi=10. To examine inhibition of apoptosis, infected cells were treated with Camptothecin (4µg). The negative control consisted of mock-infected cells and the positive control consisted of cells treated with 4µg Camptothecin. Camptothecin induces apoptosis in a dose-dependent manner [280] and has been used as a positive control for similar apoptosis assays [136].

In CEFs, Camptothecin treatment resulted in a significant increase in apoptosis levels compared to that in the negative (mock-infected/untreated) control (Fig. 3.6A and B) cells, which also underwent apoptosis, but to a lesser extent, at both time points (Fig. 3.6A and B). Although a certain level of apoptosis is expected in untreated cells, it is possible that the increased levels of apoptosis exhibited here was due to the high concentration of cells used for the assay. In CEFs at 6hrs and 12hrs all viruses alone failed to induce apoptosis (Fig. 3.6A and B). Furthermore, in CEFs treated with virus and Camptothecin, apoptosis levels were reduced to below that of the negative control for all viruses (Fig. 3.6A and B), indicating that the addition of virus inhibited the Camptothecin-induced apoptosis in these cells.

Similar observations were made in HeLa cells at 24hrs and 48hrs (Fig. 3.7C and D). In HeLa cells at 6hrs and 12hrs p.i. (Fig. 3.7A and B respectively) increased levels of apoptosis were observed in cells treated with Camptothecin and infected with FWPV, CNPV and MVA respectively, especially at 6hrs (Fig. 3.7B) and to a lesser extent at 12hrs (Fig. 3.7B).

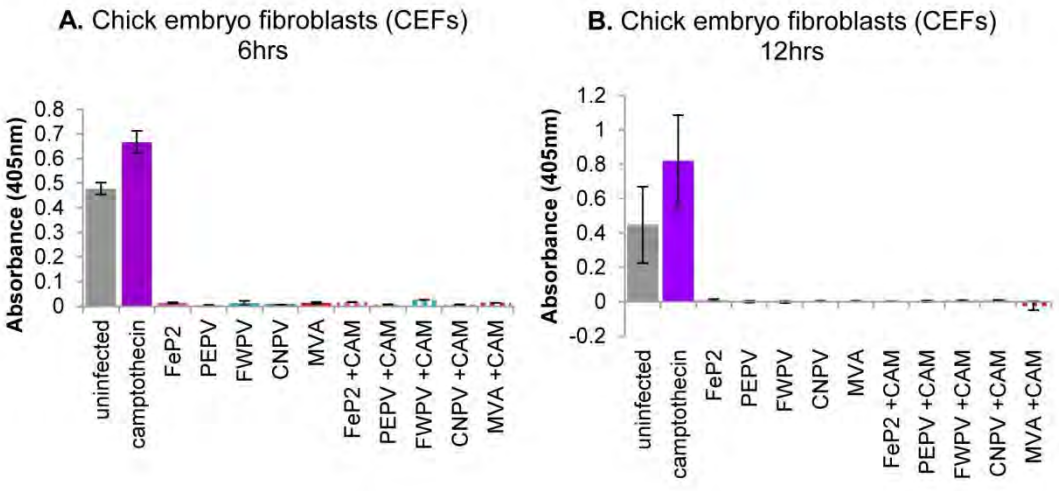


Figure 3.6 Measurement of induction and inhibition of apoptosis by FeP2 in comparison to PEPV, FWPV, CNPV and MVA in primary CEF cells at (A) 6 hours and (B) 12 hours post infection. Apoptosis was measured using the Roche Cell Death Detection ELISAPLUS kit for detection of histone-complexed DNA fragments in the cytoplasm. For induction of apoptosis, cells were infected with virus at a moi =10. For inhibition of apoptosis, cells were treated with Camptothecin (CAM) and virus simultaneously. The negative control consisted of mock-infected cells and the positive control consisted of cells treated with Camptothecin. Each sample was tested in duplicate and an average was obtained. Error bars were calculated with standard error.

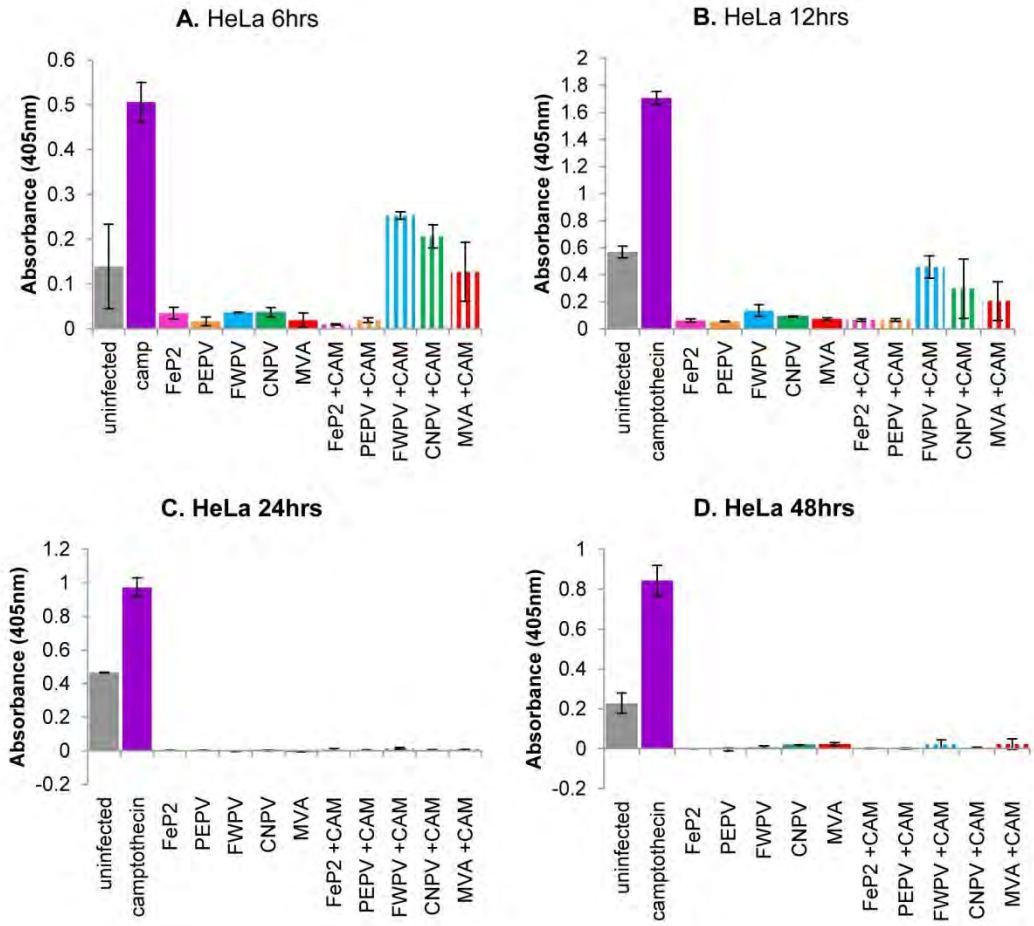


Figure 3.7 Measurement of induction and inhibition of apoptosis by FeP2 in comparison to PEPV, FWPV, CNPV and MVA in HeLa cells at (A) 6 hours, (B) 12 hours (C) 24 hours and (D) 48 hours post infection. Apoptosis was measured using the Roche Cell Death Detection ELISAPLUS kit for detection of histone-complexed DNA fragments in the cytoplasm. For induction of apoptosis, cells were infected with virus at a moi=10. For inhibition of apoptosis, cells were treated with Camptothecin (CAM) and virus simultaneously. The negative control consisted of mock-infected cells and the positive control consisted of cells treated with Camptothecin. Each sample was tested in duplicate and an average measurement was obtained. Error bars were calculated with standard error.

3.4 Discussion

Live, attenuated or host range restricted poxviruses make attractive candidates for use as vaccine vectors. It is crucial to understand the behaviour of these vectors *in vitro* and *in vivo*. In this study, a novel South African APV isolated from a Feral Pigeon (*Columba livia*) (FeP2) has been characterised in terms of growth and apoptosis in permissive and non-permissive cells, and compared to other poxviruses strains. This study will provide fundamental characterisation and a better

understanding of *in vitro* behaviour of this poxvirus which may help to determine its potential for use as a future vaccine vector.

FeP2 was shown to be permissive in CEFs with an increase in virus yield of approximately 2 logs over 5 days (Fig. 3.1). The morphogenesis of FeP2 in CEFs, as viewed by EM, was complete and followed the same sequence of events as those reported for PEPV [71], FWPV [271, 273] and CNPV [271] in CEF cells.

For both CEF (Fig. 3.1) and HeLa (Fig. 3.2) cell growth curves the 5 viruses differed in titre at day 0 – day 1. Virus stocks were titrated multiple times to ensure accuracy and for each growth curve care was taken to ensure consistent multiplicities of infection. Similarly, there were differences in FeP2 titre at day 0 – day 1, when grown in 4 different cell lines (Fig. 3.3). Here, the titre of FeP2 increased between day 0 and day 1 in the two avian cell lines, and decreased in the two mammalian cell lines. Therefore, the variable titres early after infection (day 0 - day 1) may reflect differences in viral attachment or entering.

FeP2 showed the greatest increase in virus yields compared to the other APVs; however MVA grew the most productively in CEFs (Fig. 3.1). This is not suprising as MVA was derived from the VACV Ankara strain by over 500 passages in primary CEFs [66, 68]. FeP2 is a novel virus and was initially grown on the CAMs of embryonated chicken eggs as detailed in chapter 2. Passage and subsequent adaptation of FeP2 to growth in CEFs may improve virus yields in these cells. FeP2 did not productively grow in QM7 cells of quail origin, which may be due to the fact that these cells are muscle myoblast cells and not fibroblasts like CEFs. A comparison of MVA growth in different cell lines showed that permissive cells were all fibroblastic in morphology [281].

Productive growth of FeP2 is inhibited in HeLa cells and virus yields decrease in HeLa cells over time (Fig. 3.2). FeP2 morphogenesis in HeLa cells was severely restricted with limited cytoplasmic regions of viroplasm, no characteristic viral crescents or IVs (Fig. 3.5). This was similar to the morphogenesis of CNPV observed in mammalian cell lines and of FWPV in human cell lines [271]. The production of significant levels of CPE in FeP2-infected HeLa cells (not shown) suggests that viral proteins are being produced to some extent. Alternatively the viral proteins present in the infecting virions have an effect on the cell. Reduced levels of CPE in MVA compared to NYVAC and VACV Western Reserve strain has

been attributed to the absence of certain viral proteins in MVA as a result of extensive genomic deletion [276].

Viral late gene expression occurs in MVA-infected HeLa cells and viral morphogenesis is interrupted at a late stage of the replication cycle [69]. In NYVAC infected HeLa cells, the block in viral morphogenesis occurs much earlier, prior to the formation of IVs and could be related to the absence of expression of genes encoding late structural proteins[276].

There is limited evidence of APV growth in mammalian cell lines. PEPV has been shown to undergo partial maturation in simian CV-1 (monkey kidney) cells, with morphogenesis inhibited before the stage of mature infectious particles [71]. Growth of FWPV in monkey cells (CV-1 and African green monkey Vero cells) resulted in DNA replication and early and late gene expression [275]. In the human MRC-5 cell line, FWPV early gene expression and DNA replication occurs but at lower levels than in Vero and CV-1 cells [275]. Studies of FWPV in HeLa cells showed only very low levels of genome replication and gene expression [275]. These results suggest that monkey-derived cells are more permissive to APVs than cells of human origin. VACV requires at least one of the host range genes, C7L or K1L, for replication in human MRC-5 cells but not in Vero cells suggesting that the lack of a FWPV equivalent of these genes may be responsible for the differences in gene expression in human versus monkey cell lines [28, 275].

A study comparing growth of CNPV and FWPV showed that in non-human mammalian cells (Vero and CV-1 monkey cells), CNPV replication is more restricted than for FWPV. In human MRC-5 cells, CNPV and FWPV display similar abortive replication [271]. Since CNPV has been used successfully as a vaccine vector, transgene expression in mammalian cells (despite the restricted replication) must occur at some level. Future studies on FeP2 as a vaccine vector will require the analysis and comparison of how transgene expression is affected by the block in virus replication in non-permissive cells and the correlation of this information with immunogenicity studies.

Both induction and delay/prevention of apoptosis in poxvirus vectors have been shown to improve foreign antigen expression as well as immunogenicity [134-136, 282]. Vaccine-induced apoptosis is not well understood and poxviruses encode several inhibitors of apoptosis (reviewed in chapter 1 section 1.4.4). Several

poxvirus vector candidates show differential induction of apoptosis *in vitro* in a cell-type dependent manner.

Human dendritic cells (DCs) are professional antigen-presenting cells (APC) that are important for the generation of the immune response to viral infection. In DCs, both MVA and VACV undergo abortive replication with the block occurring at the formation of intracellular viral replication centres [283]. Both VACV and MVA result in apoptosis of DCs but MVA-infected DCs undergo apoptosis earlier than VACV-infected DCs [283]. Since both MVA and VACV induce significant immune responses *in vivo*, the fact that these viruses both induce apoptosis in DCs is of interest. Cross presentation, where uninfected DCs present exogenous viral antigen acquired from dying cells, may therefore be a major mechanism of the generation of an immune response to OPV infection [283]. These results suggest that the induction of apoptosis of specific cell types may have positive implications for the generation of an enhanced immune response.

Although APC and immune cells, including DCs, macrophages and B cells, undergo apoptosis after OPV infection [284, 285], the induction of apoptosis by OPV is relatively uncommon in non-APC cell types and these cells generally demonstrate cell lysis (rather than apoptosis) in response to viral infection [286]. Investigation of virus-induced apoptosis in non-APC cell types may not accurately reflect what happens in immune cells. However, several studies have compared poxvirus vector-induced apoptosis in HeLa cells:

The ALVAC-HIV vector that expresses HIV-1 Gag, Env, and a Nef/Pol polyepitope string (vCP205) was modified (vCP1452) to encode VACV E3L and K3L genes (inhibitors of apoptosis) in an attempt to improve antigen production by this vaccine vector and so increase the potential for greater immunogenicity [134]. vCP1452 showed significantly decreased levels of apoptosis in HeLa cells and increased antigen production. However, consequent cellular immune responses elicited by this vaccine vector in human trials were substantially lower than for vCP205 [287, 288]. The reason for this is unknown; however, it is possible that the reduced apoptosis induction by vCP1452 could have contributed to the reduction in immunogenicity [136].

In contrast, NYVAC induces significant levels of apoptosis in HeLa cells, whereas MVA does not, and this induction of apoptosis is attributed to the loss of the viral

C7L gene [276]. The extent to which this difference in apoptosis induction contributes to the higher immune responses induced by the HIV-1 clade B-based vaccine candidate regimen comprising of a DNA-B prime/NYVAC-B boost compared to a DNA-B prime/MVA-B boost is unknown and remains to be delineated [282].

In our study, we show that FeP2 as well as the APVs CNPV, FWPV, PEPV and the orthopoxirus, MVA, all significantly inhibit apoptosis in permissive CEFs as well as non-permissive HeLa cells (Fig. 3.6 and 3.7). Previous studies with MVA and CNPV showed low levels of apoptosis induction in HeLa cells infected with these viruses, especially in MVA-infected cells; although virus-induced inhibition of apoptosis was not analysed [136]. The increase in apoptosis induction by CNPV seen by Zhang et al, (2007) may be strain specific. Passage and subsequent attenuation of an APV may render it less able to inhibit apoptosis. The implications of this inhibition of apoptosis in human HeLa cells are not known and future work would require further characterisation of this phenomenon in further cell types, including human DCs and other immune cells.

Although the comparison of vector-specific characteristics is necessary, the addition of transgenes into the genomes of poxvirus backbones may alter apoptosis induction. Zhang et al. (2007), showed that while empty vector CNPV induced slightly higher levels of apoptosis than MVA alone, the addition of HIV inserts (*gag/pro/env*) significantly increased MVA- and CNPV- induced apoptosis in HeLa cells to the point that vector-based differences were insignificant [136]. This highlights the necessity of investigating both vector backbone and insert effects *in vitro* and *in vivo* [136].

Here we describe the growth characteristics of a novel SA APV isolated from a Feral Pigeon (FeP2). We show the growth kinetics of this virus in permissive avian cells and non-permissive mammalian cells, and compare these to other poxviruses. Furthermore, we show that morphogenesis of FeP2 in permissive CEF cells proceeds as in other APVs, and that morphogenesis in non-permissive HeLa cells is blocked prior to the formation of immature viral particles. Lastly we show that FeP2, as well as other poxviruses, inhibit apoptosis in CEFs and HeLa cells at different time points which may have implications for immunogenicity of this virus (and the other poxviruses) if used as a vaccine vector.

Chapter 4

Genome sequence of a poxvirus isolated from a South African pigeon (FeP2)

4.1 Introduction

The pigeonpox virus, FeP2 was selected for whole genome sequencing. When grown on the CAMs of embryonated 10-11 day old chicken eggs, this virus caused greater cell proliferation and immune cell infiltration as compared to other APV isolates (chapter 2). Furthermore, based on phylogenetic analysis of four conserved genetic regions, FeP2 groups in clade A, subclade A3. FPVUS and FP9 group in clade A1 and CNPV groups in clade B1 (chapter 2). FeP2 grows productively in primary CEF cells, which is an important consideration were this virus to be used as a vaccine vector, whereby growth in cell culture is a requirement for the construction of recombinant virus.

The genomes of only three APVs have been published; a pathogenic American strain of FWPV (FPVUS) [25], an attenuated European strain of FWPV derived from European FWPV HP1 passaged over 400 times in chick embryo fibroblasts (FP9) [27], and a virulent CNPV isolated from a canary (CNPVATCC VR-111) [24]. Comparison of the CNPV and FWPV genomes reveals overall synteny in genome arrangement with similar genetic complements. They do, however, exhibit significant differences in the terminal, variable regions as well as in three internal, variable regions which is in contrast to the conservation of central genomic regions in other ChPVs. These variable regions within the conserved central region of the genomes occur near the junctions of areas that were identified in FWPV as rearranged relative to other ChPVs and contain genes involved in virus-host interactions [24, 25]. APVs are considerably divergent from other ChPV [24, 25] and may constitute a separate subfamily within the *Poxviridae* family [24, 26, 289].

FP9, FPVUS and CNPV have large genomes of 258, 280 and 365kbp encoding 238, 260 and 320 ORFs respectively [24, 25, 27]. The genomes have low G+C content (30-40%) and comprise of a central, conserved region flanked by terminal divergent regions and two identical terminal repeats (ITRs) which are covalently closed by hairpin loops [25]. The conserved central region of the genome encodes highly conserved genes encoding proteins involved in RNA and DNA synthesis, virion assembly and virus structure [24]. 90 genes have been found to be conserved amongst all ChPVs and to comprise the minimum essential genome [290]. The remainder of the genetic component of avian poxviruses is largely made up of immunomodulatory and host specific genes located in the terminal regions of the genome, that have allowed the viruses to take advantage of their unique hosts.

Laidlaw and Skinner (2004) compared virulent (FPVUS (American) and HP1 (European)) and attenuated, tissue culture adapted (FP9 (European)) FWPV strains. Comparative genomics of FWPVUS and FP9 revealed 118 differences, affecting 71 ORFs. Of these, 68 mutations affecting 25 ORFs differentiated the FWPVs geographically. The combination of adaptation to CEFs and attenuation was a result of only 50 mutations, 26 of which were single nucleotide substitutions, involving 46 ORFs that would not necessarily have been pinpointed as factors of attenuation [27]. Instead of the predicted changes in immunomodulators as the mechanism of attenuation, members of multigene families, especially those encoding ankyrin repeat proteins were seen to be the drivers of attenuation. Ankyrin repeat proteins are thought to be involved in poxvirus host-range [28], [291] and have previously been implicated in the attenuation of sheeppox virus [29]. Over 49% (137 genes) of the CNPV genome and 38% (89 genes) of the FWPV genome are comprised of genes belonging to gene families, 51 and 31 of which are ORFs containing ankyrin repeats respectively [24]. These and other differences in immunomodulatory gene families encoded by APVs may account for the extensive variability in virulence, host range and host interaction [24].

FPVUS and CNPV are significantly divergent. Comparison of CNPV and FWPV reveals variability in internal genomic regions, which is in contrast to the conservation of central genomic regions in other ChPV. Amino acid identity between ORF homologues is between 55-74% which is similar to that observed between different ChPV genera [24]. The CNPV genome contains 39 ORFs not present in FWPV, 29 of which encode unique, hypothetical proteins. CNPV contains two additional nucleotide metabolism genes (thymidylate kinase (TMPK) and the small subunit of ribonucleotide reductase), a tumour necrosis factor receptor (TNFR) (CNPV086), an IL-10-like protein (CNPV018), cellular ubiquitin (CNPV096), a protein tyrosine phosphatase (CNPV085), a thioredoxin binding protein (CNPV149), and two Rep like proteins (CNPV153 and CNPV200). FWPV contains 15 ORFs not present in CNPV, 13 of which encode hypothetical proteins. Homologues of fpv217 and fpv250 are notably absent, which are similar to ORFs from insect baculoviruses, and from avian herpesviruses and adenoviruses respectively.

There is evidence of the occurrence of integrated reticuloendotheliosis virus (REV) sequences within the genomes of some field isolates and vaccine strains of fowl and pigeon poxviruses [292-297]. REV infection, arising from the expression of the integrated provirus within FPV, has been suggested to play a role in the progression

of disease in FPV-infected birds [292]. Reticuloendotheliosis is a tumorigenic and immunosuppressive disease, and the presence of integrated REV sequences in FPV vaccines has severe implications for the poultry industry [220, 298]. FPVUS contains a 253nt remnant of REV long terminal repeat between nucleotides 232464 and 232717, however REV *env*, *gag* and *pol* genes are not present [25]. CNPV lacks sequences similar REV [24].

The FeP2 genome was sequenced and compared to the published FPVUS, FP9 and CNPV sequences.

4.2 Materials and methods

4.2.1 Viral genomic DNA isolation

FeP2 was grown on the CAMS of embryonated 10-11 day old chicken eggs as previously described (chapter 2, section 2.2.2) The virus-containing supernatant fluid was purified through a cushion of 36% sucrose in TE buffer (pH 9) and then through a gradient of 36% sucrose and 10% Dextran (in TE buffer pH 9) and resuspended in TE buffer (pH 9).

For 454 sequencing, genomic DNA was extracted using a method described previously in chapter 2, section 2.2.4 with an additional incubation with DNase I (Thermoscientific, Waltham, MA, USA) (25U/100ul virus) at 55°C for 1hr and subsequent inactivation of DNase at 80°C for 30minutes, prior to treatment with proteinase K.

For Ion Torrent sequencing, special care was taken to prevent host (chicken) chromosomal and mitochondrial DNA contamination of the viral DNA preparation. Before DNA extraction virus preparations were treated with equal volumes of Vertrel® (1,1,1,2,3,4,4,5,5,5-decafluoropentane) (DuPont, Wilmington, DE USA) which is a component that has been shown to separate virus from infected cellular debris in some viral purification methods [299]. The virus preparations were then freeze/thawed three times to lyse any remaining cells, and then treated with DNAse I prior to lysis of the virions. DNA extraction was then performed as described above.

4.2.2 DNA sequencing and bioinformatics

High quality viral DNA was subjected to high-throughput sequencing using a Roche 454 GS Junior system (Roche, Basel, Switzerland) at the University of the Western Cape. For 454 sequencing the amount of input DNA was ~380ng and not 500ng as recommended. The library was prepared with the GS Junior Titanium Rapid Library preparation kit as per manufacturer's instructions except with a 15s nebulization instead of 1 min and library preparation was checked using an Agilent Bioanalyser High sensitivity chip to ensure the average fragment length was between 500 and 900bp. For emulsion PCR the GS Junior Titanium emPCT kit (Lib-L) was used and sequencing was performed using the GS Junior Titanium Sequencing kit and PicoTiter Plate as per manufacturer‘s instructions. Primary sequence analysis was performed using the GS Junior Software version 2.5p1 and *de novo* assembly was done using GS De Novo Assembler software and CLC Genomics Workbench.

Thereafter, viral DNA was sequenced using the sequencing platform, Ion Torrent [300] Personal Genome Machine (PGM) (Life Technologies, Carlsbad, CA, USA) at the Central Analytical Facilities, Stellenbosch University. The starting amount of DNA was 400ng, and the Covaris S2 physical shearing system was used. The Ion Plus Fragment Library kit was used for library construction, which was amplified for 6 cycles prior to enrichment using the Ion OneTouch V2 Template kit. The average size of the library was calculated using the Agilent 2100 BioAnalyzer (312bp). The Ion PGM 200 Sequencing kit was used for sequencing on a 316 chip as per manufacturer‘s instructions.

To offset possible remaining host DNA contamination, we made use of a customized bioinformatics pipeline (appendix 2) as described here (performed by Anelda van der Walt). For 454 data, the SFF file was converted to FastQ in Galaxy, and filtered such that only reads shorter than 500nt and with a mean QC< 20 were included. For the Ion torrent data, primary analysis was performed in Torrent Suite version 3.2.1. Firstly, the Raw SFF file was submitted to SFFTrim (Torrent Suite 3.2.1) and reads were trimmed when the average base quality values in a window size of 10 were less than 25 (Q-value of 25). Reads shorter than 50nt were discarded. Reads were trimmed of adaptor sequences and filtered to remove polyclonal reads and trimmed to remove poor quality bases at the 3‘ end of long reads.

These two datasets were then mapped to the Chicken (Gallus gallus) genome (WASHUC2 assembly) with Newbler 2.6 to filter out possible host contamination. Read IDs that did not map to the Chicken genome were obtained from the Newbler output and these unmapped reads were extracted from the original SFF files using Linux commandline tools and SFFFile that forms part of the 454 NGS data analysis software. The resulting SFF files were used as input data for de novo assembly. De novo assembly was performed in Newbler 2.6 using default parameters, as well as in Mira (Version 3.4.0) where de novo assembly was assisted by a reference genome (FPVUS).

Roche 454 sequencing resulted in 93,654 reads with an average length of 404bp. Following filtering, only 2369 (2.5%) of these reads did not map to the chicken genome and met the quality requirements.

Ion Torrent sequencing resulted in a total of 3,239,283 reads with a mean read length of 191bp. The filtered data set contained 1,068,645 reads or 32.99% of the total raw reads. Newbler assembly resulted in 7 contigs greater than 500bp (278380 bases total). MIRA assembly also resulted in 7 contigs greater than 500bp (279145 bases total). The assemblies were merged using the Genome Assembler, Reconciliation and Merging (GARM) version 0.70 meta assembler pipeline which resulted in 3 contigs and the remaining gaps were closed by PCR and Sanger sequencing and visual inspection. Raw PGM reads were then mapped back to the draft sequence. Anelda van der Walt performed the above analysis and we acknowledge and thank her for her input.

A total of 3,142,379 raw reads mapped to the draft sequence (98% of total) resulting in an average coverage of 2130 X. Remaining gaps between contigs were closed by PCR and Sanger sequencing and raw reads were mapped back to the draft sequence using CLC Genomics workbench 4.7.2 and TMap as part of the Torrent Suite (Version 3.2.1) software.

ORFs longer than 30 amino acids with a methionine start codon (ATG) and less than 50% overlap to other ORFs were called using the CLC Genomics Workbench (CLC) ORF analysis tool as well as with an Integrated Services for Genomic Analysis (ISGA) pipeline which makes use of GLIMMER3 [301], BLAST, HMMer2 [302] and other protein coding sequence and annotation software described by Hemmerich, et al. (2010) [303]. Similarity searches including nucleotide (BLASTn)

and Protein (BLASTp) BLAST analyses were performed on every ORF and ORFs were annotated as potential genes if they shared significant sequence similarity to known viral or cellular genes (BLAST E value ≤e-5) or contained a putative conserved domain as predicted by BLASTp. ORFs were numbered from left to right, with alphabetic sub-ordering used to indicate multiple potential fragments of larger APV ORFs. As published by Hendrickson et al., (2010) [7], an ORF was annotated as a whole gene if intact at its 5‘ end with at least 80% the length of its orthologue. An ORF intact at its 5‘ end but less than 80% of its orthologue length has been annotated as a truncated gene. Any ORF that has been disrupted at its 5‘ end has been annotated as a fragmented gene, which is not expected to be transcribed and/or translated into a functional gene product [7].

Promoters described by Afonso et al. (2000) [25] were predicted using CLC motif search tool and tandem repeats were identified using TandemRepeatsFinder [304]. Multiple sequence alignments were performed with progressiveMauve [305] and Base-By-Base v2 [306]. Dotplots were done in Jdotter [307].

4.2.3 Phylogenetic analysis

Phylogenetic analyses were performed on representative amino acid sequences of DNA polymerase as well as on the concatenated amino acid sequences of 17 conserved proteins from each ChPV species, as previously described [3].

The amino acid sequences of the DNA polymerase and the 17 conserved proteins, described by Gubser (2007) [3], from representative poxvirus species were obtained from Genbank. For the 17 conserved proteins, the individual sequences of each protein were first aligned using MUSCLE [308]. Gblocks.091b [309] was then used to remove any gaps or divergent blocks according to specified criteria [309]. All 17 protein sequences for each virus were then extracted from the Gblocks output and concatenated manually. For the DNA polymerase and concatenated amino acid sequences, multiple sequence alignments were performed with MUSCLE [308]. An additional analysis was performed using the whole genome nucleotide sequences of CNPV, FPVUS, FeP2 and PEPV, which were aligned with ClustalW in Galaxy. Appropriate phylogenetic models were selected using ProtTest 2.4 [310], and phylogenetic analysis was performed in MEGA 5.10 [250] using both maximum parsimony and maximum likelihood methods with 100 bootstrap replicates.

4.2.4 Nucleotide sequence accession number

The sequence of the South African Feral Pigeonpox virus (FeP2) has been deposited in the NCBI database under GenBank accession number: KJ801920.

4.3 Results

The FeP2 genome was assembled into a contiguous sequence of 282,356bp (Genbank accession number KJ801920). Because the terminal hairpin loops were not sequenced, the left most nucleotide was arbitrarily nominated base 1.

4.3.1 Phylogenetic analysis of FeP2

Phylogenetic analysis was performed using the DNA polymerase gene, a concatenated amino acid sequence of 17 translated ORFs and the entire genomic sequence (see methods). All three analyses produced equivalent tree topologies (Fig. 4.1). FeP2 groups with FWPV (clade A) separately from CNPV (Clade B). Because of the limited number of whole APV genome sequences, it was not possible to determine the subclade grouping of FeP2.

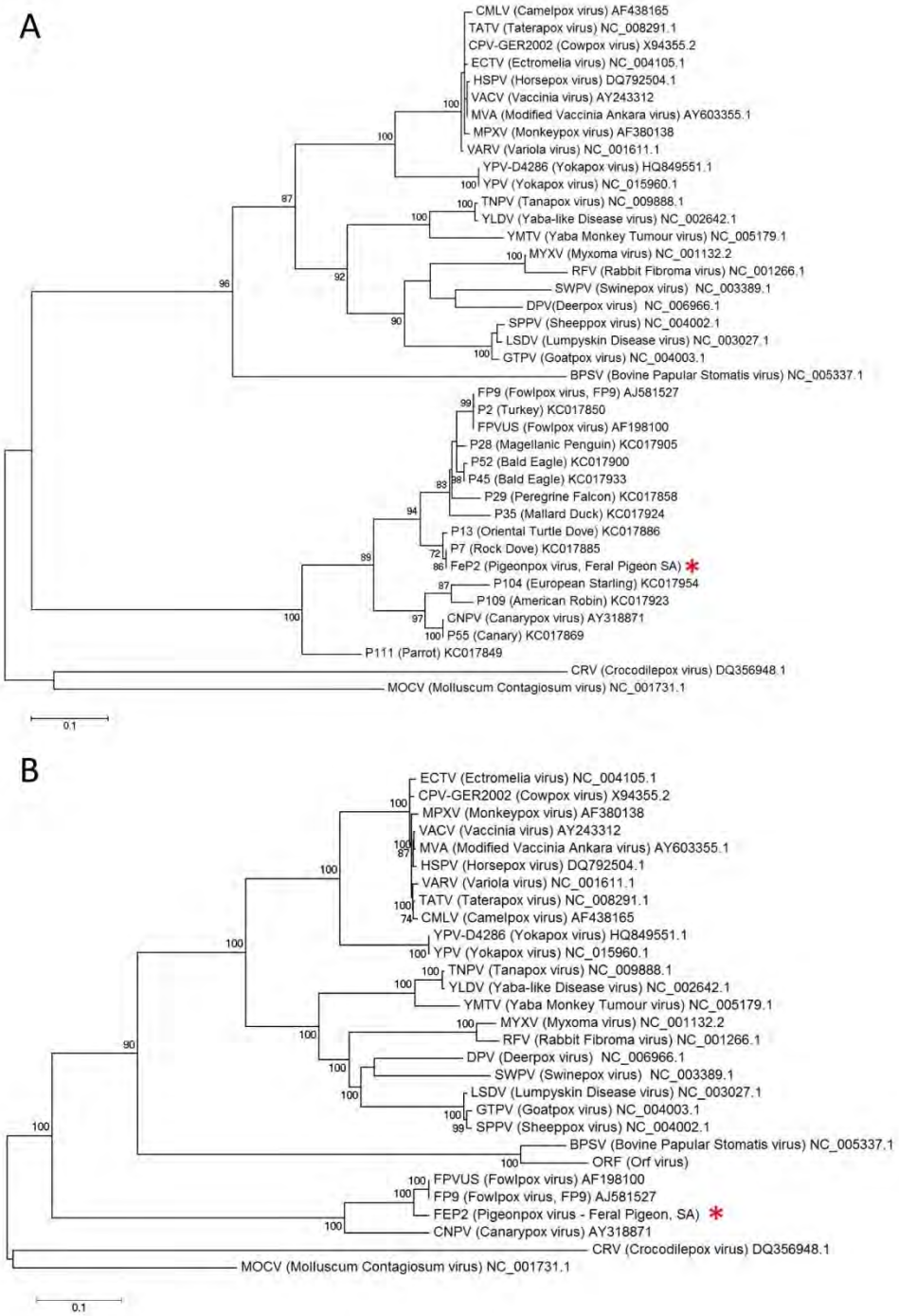


Figure 4.1 Maximum likelihood phylogenetic trees based on the amino acid sequence alignment of A) the DNA polymerase genes and B) 17 concatenated genes from selected poxviruses. Multiple sequence alignment was performed with MUSCLE and the appropriate phylogenetic model was selected using ProtTest 2.4. The maximum likelihood phylogenetic tree based on the JTT matrix-based model (121) with 100 bootstrap replicates was constructed in MEGA 5.10. Discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated.

4.3.2 Overall genetic comparison of FeP2 to FWPV and CNPV.

The three genomic sequences of FeP2, FWPV and CNPV were compared for nucleotide percentage identity. FeP2 is more closely related to FWPV (84.0%) than to CNPV (63.4%). Dot plot analysis reveals a large deletion of 16kb in FeP2 compared to FWPV (Fig. 4.2 A). Like the other APVs, FeP2 has an A/T rich genome, with 70.5% A+T content.

4.3.3 Inverted terminal repeats (ITRs)

The FeP2 genome, like most other poxviruses, contains two identical inverted repeated sequences at its termini (ITRs). These are 4682bp in length which are shorter than the ITRs of the other sequenced APVs (FPVUS: 9520bp, FP9: 10158bp and CNPV: 6491bp). Like FPVUS and CNPV, the A+T content within the two ITRs is lower than average at 64.8%. At the terminal ends of each ITR in FeP2 exists a 257bp repeat region with 3.1 and 3.5 copies respectively of a 34bp and 55bp tandem repeat. The 34bp repeats occur between nt 7-111 and 282090-282279, and the 55bp repeats are found between nt 75-264 and 282243-282347. FPVUS contains a 1.7kb region with 42 copies of a 31-32bp tandem repeat (between nt198-1853, and 286703-288340) [25]. CNPV contains at least 31, 9, and 7 copies of 17-, 41-, and 48-bp tandem repeats respectively within the terminal regions of each ITR [24]. The repeated sequences are unique to the different APVs. Three intact ORFs, orthologous to fpv001 (fep001) and fpv002 (fep003) and to cnpv021 (fep002, fpv246), are present in the ITRs.

4.3.4 Overall arrangement of ORFs in genome

The genomic arrangement of ORFs in FeP2 is shown in Figure 4.3 and has been compared to FPVUS, FP9 and CNPV in Figure 4.4. Clearly, there is conservation amongst the 4 sequenced APVs, but, at the same time, there are major differences between them. Unlike the OPVs the APVs exhibit variation within the central core region, where blocks of APV genes have been inverted and/or transposed, suggesting that this genus is the most divergent of the ChPVs [3, 24, 25]. Comparison of gene orthologues indicate overall genomic synteny between FeP2 and FPVUS and genomic organisation is similar to that of other sequenced ChPVs [10, 25, 246]. Table 4.1 lists all ORFs potentially coding for proteins of >30 amino acids for FeP2, and Figures 4.3 and 4.4 represent these graphically.

FeP2 contains 271 ORFs which have been annotated as putative genes. Orthologues of FPVUS genes as well as other genes have been determined (Table 4.1, Fig. 4.3). These ORFs represent an approximate coding density of 82.5% and encode proteins between 34 and 1937 amino acids (Table 4.1). Of these 271 ORFs, 11 have been annotated as truncated genes (Table 4.2). A further 36 of these ORFs are fragmented forms of 28 larger orthologous genes (Table 4.2). Therefore a total of 224 ORFs have been annotated as full length putative genes.

Relative to the FPVUS genome, FeP2 has 36 deleted ORFs (Table 4.2). FeP2 contains 34 inserted ORFs relative to FPVUS, comprising 12 fragments of larger genes, 2 truncated genes and 20 intact genes (Table 4.3). 5 of the intact genes do not have any functional orthologue in FPVUS (Table 4.1). Between FeP2 and FWPV, the most conserved ORF is the orthologue of fpv103 (fep105 and VACV F17R) which encodes the DNA binding virion core phosphoprotein and shares 100% amino acid identity amongst the three viruses.

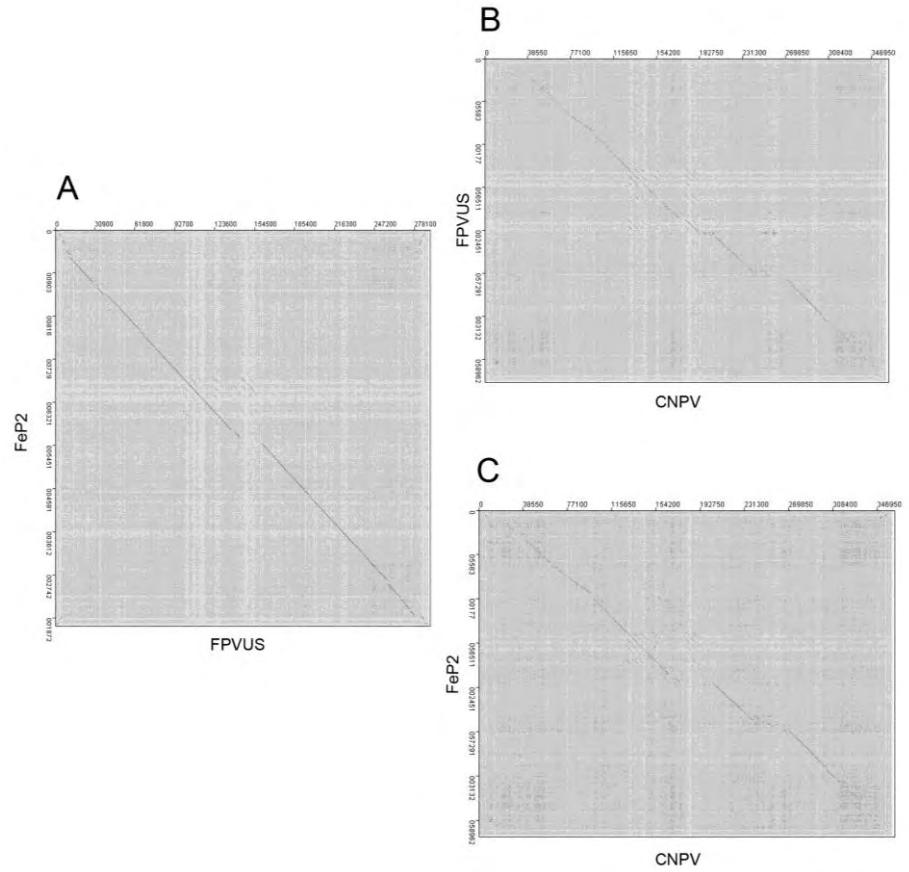


Figure 4.2 Dotplot comparisons of FeP2, FPVUS and CNPV genomic sequences. A) Dotplot comparing FPVUS (horizontal) and FeP2 (vertical). B) Dotplot comparing CNPV (horizontal) and FPVUS (vertical). C) Dotplot comparing CNPV (horizontal) and FeP2 (vertical). Dotplots were constructed using Jdotter (Brodie et al., 2004). Major deletions can be seen as breaks in the diagonal line. Lines perpendicular to the main diagonal in the top-right and bottom-left corners indicate the inverted terminal repeats (ITRs). The short diagonal lines, parallel to the main diagonal and near the centre of the plots, represent the members of the variola virus B22R family.

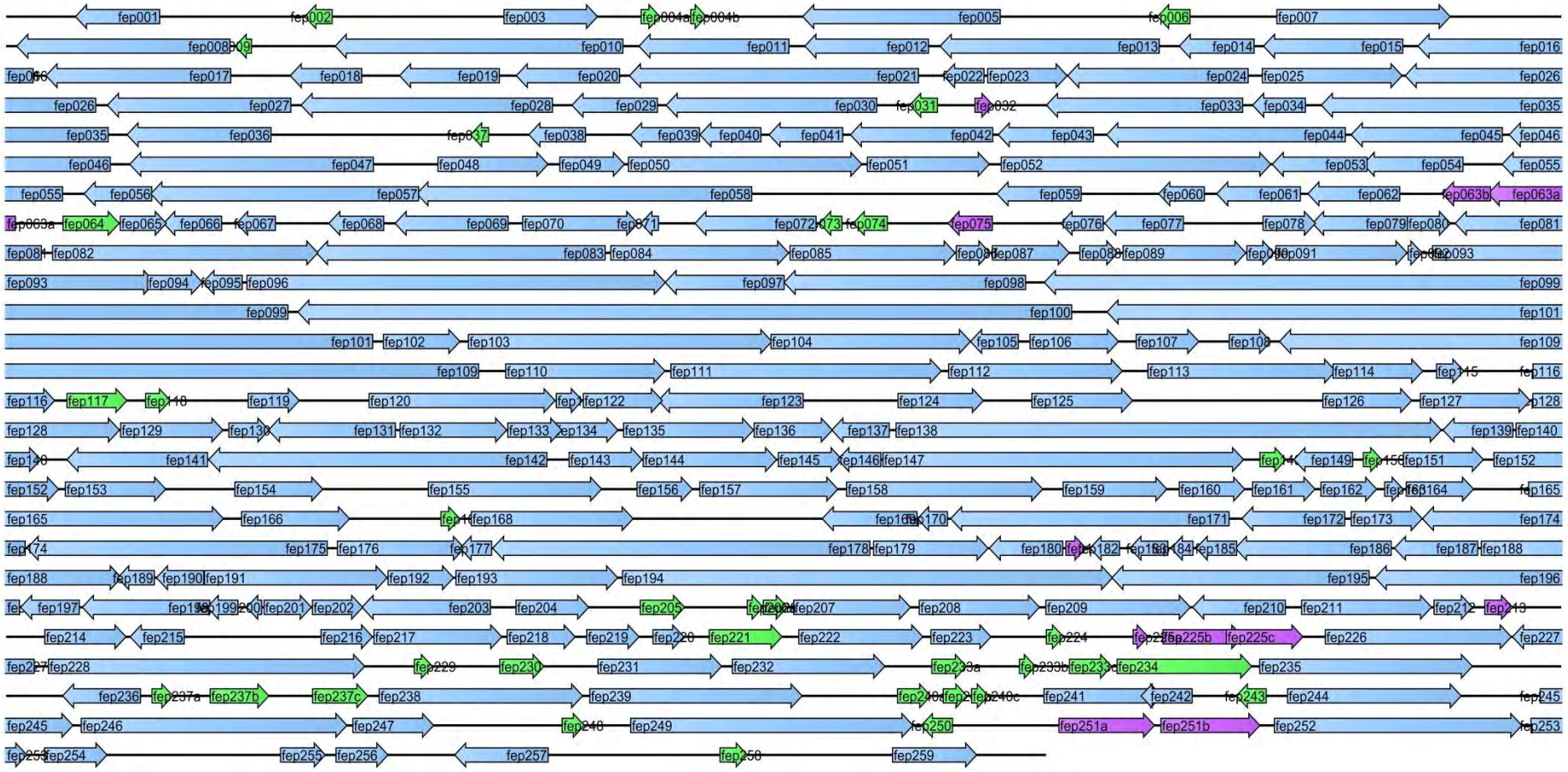


Figure 4.3 Genome map of FeP2. Intact open reading frames (ORFs) are depicted in blue. Truncated ORFs are depicted in purple and fragmented genes are in green. An ORF was annotated as a whole gene if intact at its 5‘ end with at least 80% the length of its orthologue. Truncated genes were annotated as such if intact at its 5‘ end but less than 80% of its orthologue and fragmented genes were genes that were disrupted at the 5‘ end.

FeP2 lacks sequence similar to the REV observed in FPVUS and other APV strains [25, 292-297].

As described above, the FeP2 genome is closely related and syntenic to the FWPV genome overall (Fig. 4.4); however, differences in gene content are observed. Of particular note is the large deletion in FeP2 relative to FPVUS (Fig. 4.2A).The deleted region corresponds to the FPVUS genes fpv121 (cc-chemokine family), fpv122 (VACV B22R gene), fpv123 (VACV B22R gene), fpv124 (N1R/p28 family gene) and fpv125 (V-type Ig domain family gene).

4.3.5 Conserved genes

There are 181 genes that are conserved amongst the four sequenced APVs, which have been highlighted in blue in Figure 4.4. These include genes that lie within the more variable terminal regions of the genome. These APV core genes were defined as such if intact orthologues of the gene were present at least once in all five APV sequences. The 181 APV core genes include the 90 core genes conserved in all ChPVs which are involved in essential functions such as replication, transcription and virion assembly [3, 8] (indicated in bold and italic in Table 4.1). There are an additional 91 genes conserved in all APVs with a variety of different functions (indicated in bold in Table 4.1).

4.3.6 Gene families

APVs contain extensive gene families, which vary greatly between different species. In CNPV, these comprise over 49% of the genome, whereas they encompass 38% of the FWPV genome [24, 25]. In CNPV and FWPV, these gene families account for much of the variation between the two genomes [24]. Intact members of gene families comprise 33% of the FeP2 genome. Table 4.4 summarises the differences in the number of intact gene family proteins found in the five sequenced APV genomes. FeP2 is noticeably different in its reduced number of ankyrin repeat proteins and C-type lectin gene families; and in the absences of genes encoding β-NGF and IL-18 binding protein.

Table 4.1 FeP2 open reading frames

FEP2 ORF	length (aa)	FeP2 status	Homolog [α]	Homolog length (aa)	% amino acid identity	VACV ORF	Description/ putative function
1	200	I	fpv001	205	77.18	A40R	C-type lectin family
2	66	F	cnpv021/fpv246	1585	5.61		Hypothetical protein Aasi_1435 [Candidatus Amoebophilus asiaticus 5a2]
3	**222**	**I**	**fpv002**	**222**	**91.03**		**Hypothetical protein**
4a	46	F	cnpv006/fpv002.5	182	14.75		Hypothetical protein
4b	38	F	cnpv006/fpv002.5	182	9.74		Hypothetical protein
5	467	I	cnpv320/fpv017	465	83.19		Ig-like domain protein
6	79	F	cnpv309/fpv241	196	14.14	M1L	Ankyrin repeat family
7	**410**	**I**	**fpv006**	**418**	**84.25**	**C10L**	**C4L/C10L-like gene family protein**
8	504	I	cnpv015/fpv162	528	42.11	B4R	Ankyrin repeat family
9	40	F	TVAG_011430	732	3.69		Ankyrin repeat family [Trichomonas vaginalis G3]
10	680	I	fpv244	668	31.66		Ankyrin repeat family
11	**355**	**I**	**fpv010**	**355**	**84.51**		**Serpin family**
12	**293**	**I**	**fpv011**	**278**	**74.4**		**α-SNAP**
13	518	I	fpv246	592	30.22		
14	178	I	IL10 (Ficedula albicollis[α])	177	28.26		Interleukin-10
15	**330**	**I**	**fpv012**	**331**	**76.2**		**Ankyrin repeat family**
16	403	I	cnpv028/fpv240	362	34.87	B4R	Ankyrin repeat family
17	437	I	fpv014	437	86.73		Ankyrin repeat family
18	170	I	fpv015	177	75.71		Hypothetical protein
19	**237**	**I**	**fpv016**	**238**	**79.83**		**Hypothetical protein**
20	**245**	**I**	**fpv017**	**245**	**76.92**		**V-type Ig domain**
21	**683**	**I**	**fpv018**	**700**	**83.88**		**Ankyrin repeat family**
22	**97**	**I**	**fpv019**	**104**	**56.25**		**Hypothetical protein**
23	189	I	cnpv037	171	44.04		Hypothetical protein (fragment in FPV)
24	**427**	**I**	**fpv020**	**426**	**87.12**	**C10L**	**C4L/C10L-like family**
25	**331**	**I**	**fpv021**	**320**	**78.25**		**G-protein-coupled receptor family**
26	**581**	**I**	**fpv022**	**578**	**84.88**		**Ankyrin repeat family**
27	**434**	**I**	**fpv023**	**434**	**90.32**		**Ankyrin repeat family**
28	**594**	**I**	**fpv024**	**596**	**89.95**		**Ankyrin repeat family**
29	**203**	**I**	**fpv025**	**203**	**86.7**		**Hypothetical protein**
30	498	I	cnpv044/fpv024	480	38.92	B4R	Ankyrin repeat family
31	67	F	fpv026	436	10.78		Ankyrin repeat family
32	44	T	fpv028	180	15		hypothetical protein
33	464	I	cnpv046/fpv024	450	48.39		Ankyrin repeat family
34	**126**	**I**	**fpv029**	**124**	**89.68**		**Hypothetical protein**
35	**808**	**I**	**fpv030**	**817**	**82.62**		**Alkaline phosphodiesterase**
36	**341**	**I**	**fpv031**	**341**	**91.79**		**Ankyrin repeat family**
37	44	F	fpv034	415	7.45		Ankyrin repeat family
38	135	I	fpv035	135	96.03		Hypothetical protein
39	**164**	**I**	**fpv037**	**162**	**68.29**		**Hypothetical protein**
40	**145**	**I**	**fpv038**	**145**	**89.66**	**F2L**	**dUTP pyrophosphatase**
41	**175**	**I**	**fpv039**	**175**	**81.14**		**Bcl-2**
42	**337**	**I**	**fpv040**	**337**	**91.39**		**Serpin family**
43	**226**	**I**	**fpv041**	**206**	**57.83**		**Hypothetical protein**
44	**564**	**I**	**fpv043**	**564**	**89.89**	**A50R**	**DNA ligase**
45	**358**	**I**	**fpv044**	**358**	**91.06**		**Serpin family**
46	**370**	**I**	**fpv046**	**370**	**82.75**	**A44L**	**Hydroxysteroid dehydrogenase**
47	**576**	**I**	**fpv047**	**612**	**74.39**	**A39R**	**Semaphorin**
48	**261**	**I**	**fpv048**	**261**	**93.87**		**GNS1/SUR4**
49	***154***	***I***	***fpv049***	***154***	***94.81***	***A1L***	***Late transcription factor VLTF2***
50	***552***	***I***	***fpv050***	***552***	***97.1***	***D13L***	***Rifampicin resistance, N3L protein***
51	***289***	***I***	***fpv051***	***289***	***95.16***	***D12L***	***mRNA capping enzyme***
52	***637***	***I***	***fpv052***	***637***	***95.6***	***D11L***	***NPH-1 transcription termination factor***
53	***225***	***I***	***fpv053***	***225***	***95.56***	***D10L***	***muT motif; gene expression regulation***
54	***237***	***I***	***fpv054***	***231***	***92.41***	***D9R***	***muT motif***
55	274	I	fpv055	275	64.13		V-type Ig Domain
56	***161***	***I***	***fpv056***	***161***	***96.89***	***D7R***	***RNA polymerase subunit RPO18***
57	***633***	***I***	***fpv057***	***633***	***98.58***	***D6R***	***Early transcription factor VETFs***
58	***791***	***I***	***fpv058***	***791***	***97.98***	***D5R***	***NTPase; DNA replication***
59	**200**	**I**	**fpv060**	**188**	**73.5**		**CC chemokine family**
60	109	I	fpv061	129	66.41		CC chemokine family
61	199	I	cnpv232/fpv060	204	30.23		CC chemokine family
62	***218***	***I***	***fpv062***	***218***	***97.71***	***D4R***	***Uracil DNA glycosylase***
63a	93	T	fpv063	400	39.91		

63b	134	T	fpv063	400	21.75		
64	134	F	fpv064	200	61.19		Glutathione peroxidase
65	**110**	**I**	**fpv065**	**111**	**80.18**		**Hypothetical protein**
66	**138**	**I**	**fpv066**	**122**	**73.19**		**Hypothetical protein**
67	**93**	**I**	**fpv067**	**90**	**87.1**		**HT motif family**
68	**131**	**I**	**fpv068**	**133**	**66.2**		**Hypothetical protein**
69	***268***	***I***	***fpv069***	***270***	***92.59***	***D3R***	***Virion protein***
70	**273**	**I**	**fpv070**	**273**	**84.98**		**T10 gene product**
71	43	I	cnpv095/fpv070.5	45	53.33		Hypothetical protein
72	**287**	**I**	**fpv071**	**289**	**88.93**		**Hypothetical protein**
73	54	F	cnpv098	80	27.71		Hypothetical protein
74	82	F	fpv072	186	39.25		Beta-Nerve growth factor
75	106	T	fpv073	174	37.93		Interleukin (IL18) binding protein
76	**101**	**I**	**fpv074**	**104**	**77.88**		**Hypothetical protein**
77	**187**	**I**	**fpv075**	**199**	**82.41**		**N1R/p28 family**
78	***125***	***I***	***fpv077***	***125***	***95.2***	***G4L***	***Gutaredoxin***
79	***225***	***I***	***fpv079***	***225***	***94.22***	***G2R***	***Putative elongation factor***
80	***103***	***I***	***fpv078***	***103***	***92.23***	***G3L***	***Hypothetical protein***
81	**335**	**I**	**fpv080**	**363**	**69.04**		**Transforming Growth Factor β (TGF-B)**
82	***627***	***I***	***fpv081***	***626***	***94.42***	***G1L***	***Metalloprotease***
83	***682***	***I***	***fpv082***	***682***	***93.84***	***I8R***	***DNA/RNA helicase/NPH-11***
84	***421***	***I***	***fpv083***	***421***	***96.67***	***I7L***	***Virion core proteinase***
85	***391***	***I***	***fpv084***	***390***	***94.37***	***I6L***	***DNA-binding protein***
86	***81***	***I***	***fpv085***	***81***	***90.12***	***I5L***	***IMV membrane protein***
87	**183**	**I**	**fpv086**	**183**	**86.89**	**J2R**	**Thymidine kinase**
88	**82**	**I**	**fpv087**	**91**	**92.31**		**HT motif family**
89	***291***	***I***	***fpv088***	***290***	***94.85***	***I3L***	***DNA binding phosphoprotein***
90	***65***	***I***	***fpv089***	***65***	***95.38***	***I2L***	***Hypothetical protein***
91	***311***	***I***	***fpv090***	***311***	***98.39***	***I1L***	***Virion protein***
92	34	I	fpO3L	34	88.24	O3L	Orthologue of vaccinia O3L & MC043.1L
93	**656**	**I**	**fpv091**	**656**	**89.48**	**O1L**	**Hypothetical protein**
94	**131**	**I**	**fpv092**	**131**	**93.13**	**E11L**	**Hypothetical protein**
95	***94***	***I***	***fpv093***	***94***	***92.55***	***E10R***	***Sulfhydryl oxidase ERV1***
96	***988***	***I***	***fpv094***	***988***	***92.42***	***E9L***	***DNA polymerase***
97	***282***	***I***	***fpv095***	***272***	***89.36***	***E8R***	***Hypothetical protein***
98	***571***	***I***	***fpv096***	***571***	***95.8***	***E6R***	***Hypothetical protein***
99	1885	I	fpv097	1912	85.45		VARV B22R family
100	**1826**	**I**	**fpv098**	**1802**	**95.47**		**VARV B22R family**
101	**1937**	**I**	**fpv099**	**1949**	**84.66**		**VARV B22R family**
102	***182***	***I***	***fpv100***	***182***	***96.7***	***E4L***	***RNA pol subunit RPO30***
103	***717***	***I***	***fpv101***	***717***	***92.33***	***E2L***	***Hypothetical protein***
104	***472***	***I***	***fpv102***	***472***	***98.09***	***E1L***	***Poly(A) polymerase large subunit, PAP-L***
105	***114***	***I***	***fpv103***	***114***	***100***	***F17R***	***DNA binding virion core phosphoprotein***
106	**210**	**I**	**fpv104**	**210**	**81.43**		**Hypothetical protein**
107	***149***	***I***	***fpv105***	***148***	***95.97***	***F15L***	***Hypothetical protein***
108	**99**	**I**	**fpv106**	**99**	**66**		**Conserved hypothetical protein**
109	**1780**	**I**	**fpv107**	**1777**	**85.35**		**VARV B22R family**
110	***377***	***I***	***fpv108***	***377***	***94.69***	***F13L***	***Virion envelope protein***
111	***639***	***I***	***fpv109***	***630***	***84.98***	***F12L***	***Virion release protein***
112	**142**	**I**	**fpv110**	**451**	**75**	**F11L**	
113	***444***	***I***	***fpv111***	***444***	***96.85***	***F10L***	***SER/THR protein kinase (virus assembly)***
114	***213***	***I***	***fpv112***	***213***	***94.37***	***F9L***	***Hypothetical protein***
115	**66**	**I**	**fpv113**	**66**	**95.45**		
116	**183**	**I**	**fpv114**	**183**	**92.35**		**HAL3 domain**
117	143	F	cnpv011/fpv246	586	8.53		Ankyrin repeat family
118	60	F	cnpv004/fpv246	514	5.25		
119	122	I	fpv116	120	81.15		CC-chemokine family
120	***440***	***I***	***fpv117***	***440***	***91.59***	***G5R***	***Hypothetical protein***
121	***63***	***I***	***fpv118***	***63***	***96.83***	***G5.5R***	***RNA pol subunit RPO7***
122	***188***	***I***	***fpv119***	***188***	***95.21***	***G6R***	***Hypothetical protein***
123	***343***	***I***	***fpv120***	***343***	***96.5***	***G7L***	***Virion core protein***
124	203	I	cnpv012/fpv229	189	31.07		Hypothetical protein
125	239	I	cnpv224	239	55.69		hypothetical protein
126	211	I	cnpv170	212	81.6		Thymidylate Kinase
127	***260***	***I***	***fpv126***	***260***	***98.85***	***G8R***	***VLTF-1***
128	***336***	***I***	***fpv127***	***336***	***92.56***	***G9R***	***Myristylated protein***
129	***243***	***I***	***fpv128***	***243***	***95.88***	***L1R***	***Myristylated protein***
130	***96***	***I***	***fpv129***	***96***	***86.46***	***L2R***	***Hypothetical protein***
131	***301***	***I***	***fpv130***	***301***	***93.69***	***L3L***	***Hypothetical protein***
132	***253***	***I***	***fpv131***	***253***	***94.47***	***L4R***	***DNA binding virion core VP8***

133	***129***	***I***	***fpv132***	***129***	***93.8***	***L5R***	***Putative membrane protein***
134	***148***	***I***	***fpv133***	***148***	***96.62***	***J1R***	***Hypothetical protein***
135	***308***	***I***	***fpv134***	***308***	***94.81***	***J3R***	***PolyA polymerase (PAPs)***
136	***186***	***I***	***fpv135***	***186***	***94.62***	***J4R***	***RNA pol Subunit RPO22***
137	***137***	***I***	***fpv136***	***137***	***91.24***	***J5L***	***Membrane protein***
138	***1287***	***I***	***fpv137***	***1287***	***96.81***	***J6R***	***RNA pol Subunit RPO147***
139	***166***	***I***	***fpv138***	***166***	***94.58***	***H1L***	***Protein tyrosine Phosphatase***
140	***190***	***I***	***fpv139***	***190***	***96.84***	***H2R***	***Hypothetical protein***
141	***333***	***I***	***fpv140***	***327***	***87.39***	***H3L***	***Virion env protein(p35)***
142	***799***	***I***	***fpv141***	***798***	***96.37***	***H4L***	***RNA polymerase associated protein RAP94***
143	***174***	***I***	***fpv142***	***174***	***89.66***	***H5R***	***VLTF-4***
144	***316***	***I***	***fpv143***	***316***	***96.2***	***H6R***	***DNA topoisomerase***
145	***152***	***I***	***fpv144***	***152***	***92.11***	***H7R***	***Putative 17 kDa protein***
146	**103**	**I**	**fpv145**	**103**	**84.47**		
147	***852***	***I***	***fpv146***	***851***	***94.01***	***D1R***	***mRNA capping enzyme, large subunit***
148	63	F	fpv147	104	51.92		HT motif family
149	***140***	***I***	***fpv148***	***139***	***90***	***D2L***	***virion protein***
150	45	F	Erysipelotrichaceae bacterium 3_1_53	112	16.07		Hypothetical protein [Erysipelotrichaceae bacterium 3_1_53]
151	**190**	**I**	**fpv149**	**186**	**84.74**		**Hypothetical protein**
152	**284**	**I**	**fpv150**	**276**	**81.69**		**N1R/p28 gene family protein**
153	**238**	**I**	**fpv151**	**235**	**84.1**		**dCK**
154	209	I	fpv153	208	77.25		Hypothetical protein
155	**410**	**I**	**fpv155**	**408**	**85.85**		**N1R/p28 family**
156	132	I	fpv156	132	87.88		HT motif family
157	**327**	**I**	**fpv157**	**311**	**78.35**		**N1R/p28 family**
158	**464**	**I**	**fpv158**	**464**	**95.04**		**Photolyase**
159	246	I	fpv159	241	83.74		N1R/p28
160	156	I	fpv160	156	94.23		Hypothetical protein
161	**149**	**I**	**fpv161**	**157**	**83.44**		**N1R/p28 family**
162	133	I	cnpv210/fpv075	131	43.7		N1R/p28 family
163	45	I	cnpv211/fpv161.5	54	36.36		Hypothetical protein
164	160	I	cnpv212/fpv124	176	53.41		N1R/p28 family
165	**591**	**I**	**fpv162**	**603**	**78.31**		**Ankyrin repeat family**
166	256	I	fpv163	263	76.05		N1R/p28 family
167	46	F	fpv162	603	3.32		Ankyrin repeat family
168	**383**	**I**	**fpv164**	**383**	**67.36**		**Hypothetical protein**
169	***225***	***I***	***fpv165***	***225***	***95.11***	***A2L***	***Late transcription factor VLTF-3***
170	***72***	***I***	***fpv166***	***72***	***95.83***	***A2.5L***	***Virus redox protein***
171	***658***	***I***	***fpv167***	***658***	***98.33***	***A3L***	***Virion core protein P4b***
172	***244***	***I***	***fpv168***	***288***	***63.09***	***A4L***	***Immunodominant virion protein***
173	***169***	***I***	***fpv169***	***167***	***97.04***	***A5R***	***RNA pol subunit RP019***
174	***374***	***I***	***fpv170***	***375***	***94.39***	***A6L***	***Hypothetical protein***
175	***709***	***I***	***fpv171***	***709***	***97.18***	***A7L***	***Early transcription factor large subunit, VETF-L***
176	***301***	***I***	***fpv172***	***301***	***97.67***	***A8R***	***Intermediate transcription factor VITF-3***
177	***76***	***I***	***fpv173***	***76***	***98.68***	***A9L***	***Hypothetical protein***
178	***891***	***I***	***fpv174***	***891***	***96.07***	***A10L***	***Virion core protein P4a***
179	***272***	***I***	***fpv175***	***274***	***96.35***	***A11R***	***Hypothetical protein***
180	***175***	***I***	***fpv176***	***171***	***88***	***A12L***	***Virion protein***
181	49	T	fpv177	68	61.76		Hypothetical protein
182	***71***	***I***	***fpv178***	***71***	***88.73***	***A13L***	***Virion protein***
183	***91***	***I***	***fpv179***	***91***	***94.51***	***A14L***	***Virion envelope protein***
184	***53***	***I***	***fpv179.1***	***54***	***98.11***	***a14.5***	***Virion envelope protein***
185	***97***	***I***	***fpv180***	***97***	***96.91***	***A15L***	***Hypothetical protein***
186	***369***	***I***	***fpv181***	***369***	***91.87***	***A16L***	***Putative mystirilated membrane protein***
187	***198***	***I***	***fpv182***	***198***	***97.98***	***A17L***	***Phosphorylated virion membrane protein***
188	***462***	***I***	***fpv183***	***462***	***95.89***	***A18R***	***DNA helicase (transcription elongation)***
189	***88***	***I***	***fpv184***	***88***	***93.18***	***A19L***	***Hypothetical protein***
190	***113***	***I***	***fpv186***	***113***	***92.04***	***A21L***	***Hypothetical protein***
191	***432***	***I***	***fpv185***	***433***	***92.84***	***A20R***	***Processivity factor***
192	***156***	***I***	***fpv187***	***156***	***91.03***	***A22R***	***Hypothetical protein***
193	***383***	***I***	***fpv188***	***383***	***94.26***	***A23R***	***Intermediate transcription factor VITF-3***
194	***1157***	***I***	***fpv189***	***1161***	***97.76***	***A24R***	***RNA pol subunit RPO132***
195	***608***	***I***	***fpv190***	***620***	***87.18***	***A25L***	***A-type inclusion protein***
196	***472***	***I***	***fpv191***	***474***	***91.77***	***A26L***	***A-type inclusion protein***
197	***140***	***I***	***fpv192***	***141***	***95.74***	***A28L***	***Hypothetical protein***

198	***302***	***I***	***fpv193***	***302***	***89.4***	***A29L***	***RNA pol subunit RPO35***
199	***74***	***I***	***fpv194***	***74***	***97.3***	***A30L***	***Hypothetical protein***
200	37	I	fp9.194.1		81.58		A30.5L orthologue
201	**113**	**I**	**fpv195**	**113**	**91.15**	**A31R**	**Hypothetical protein**
202	**120**	**I**	**fpv196**	**120**	**81.67**		**Hypothetical protein**
203	***304***	***I***	***fpv197***	***301***	***92.11***	***A32L***	***Virion assembly protein***
204	***173***	***I***	***fpv198***	***173***	***92.49***	***A34R***	***C-type lectin-like protein***
205	106	F	fpv199	219	38.81		V-type Ig Domain
206a	39	F	fpv200	265	10.94		V-type Ig domain
206b	58	F	fpv200	265	17.36		V-type Ig domain
207	**277**	**I**	**fpv201**	**283**	**87.28**		**Hypothetical protein**
208	**285**	**I**	**fpv203**	**285**	**83.51**		**Tyrosine protein kinase**
209	**342**	**I**	**fpv204**	**342**	**90.64**		**Serpin family**
210	**220**	**I**	**fpv205**	**218**	**82.27**		**Hypothetical protein**
211	**308**	**I**	**fpv206**	**308**	**87.99**		**G-protein-coupled receptor family**
212	**92**	**I**	**fpv207**	**100**	**77**		**Hypothetical protein**
213	67	T	CNPV279	169	13.64		Beta-NGF-like protein
214	**213**	**I**	**fpv208 (cnpv281)**	**214**	**40.19**		**Hypothetical protein**
215	**139**	**I**	**fpv209**	**130**	**74.62**		**HT motif family**
216	**123**	**I**	**fpv211**	**125**	**80.95**	**C11R**	**Epidermal Growth Factor-like protein**
217	**303**	**I**	**fpv212**	**303**	**93.01**	**B1R**	**Serine/threonine protein kinase**
218	**162**	**I**	**fpv213**	**162**	**90.74**		**Hypothetical protein**
219	**125**	**I**	**fpv214**	**124**	**76.98**		**Putative 13.7 kDa protein**
220	**74**	**I**	**fpv215**	**74**	**94.59**		**Hypothetical protein**
221	173	F	LOC100486298	959	4.7		Hypothetical protein LOC100486298 [Xenopus (Silurana) tropicalis]
222	**294**	**I**	**fpv216**	**296**	**84.46**		**Ankyrin repeat family**
223	143	I	Tanapox 67R	178	26.52		67R Tanapox host range protein
224	42	F	fpv217	328	9.45		Hypothetical protein
225a	37	T	fpv218	461	6.29		Ankyrin repeat family
225b	190	T	fpv218	461	29.87		Ankyrin repeat family
225c	108	T	fpv218	461	28.63		Ankyrin repeat family
226	**440**	**I**	**fpv219**	**434**	**84.2**		**Ankyrin repeat family**
227	183	I	fpv221	183	88.52	A47L	A47L homolog
228	**747**	**I**	**fpv222**	**747**	**82.89**		**Ankyrin repeat family**
229	44	F	fpv223	141	21.99		Ankyrin repeat family
230	106	F	cnpv298/fpv223-225	571	8.58		Ankyrin repeat family
231	**293**	**I**	**fpv226**	**292**	**88.74**	**B1R**	**Serine/threonine protein kinase**
232	**361**	**I**	**fpv227**	**361**	**88.92**		**Ankyrin repeat family**
233a	83	F	fpv228	525	12.38		Ankyrin repeat family
233b	39	F	fpv228	525	5.33		Ankyrin repeat family
233c	101	F	fpv228	525	14.29		Ankyrin repeat family
234	319	F	Orientia tsutsugamushi str. Boryong/303	500	21.37		
235	503	I	cnpv301/fpv233	527	37.13		Ankyrin repeat family
236	185	I	fpv229	180	80		Hypothetical A47L-like protein
237a	51	F	cnpv303/fpv230-231	256	2.5		Ankyrin repeat family
237b	140	F	cnpv303/fpv230-231	256	15		Ankyrin repeat family
237c	132	F	cnpv303/fpv230-231	256	11.16		Ankyrin repeat family
238	**481**	**I**	**fpv232**	**482**	**85.68**		**Ankyrin repeat family**
239	**502**	**I**	**fpv233**	**512**	**85.55**		**Ankyrin repeat family**
240a	80	F	fpv234	428	15.89		Ankyrin repeat family
240b	57	F	fpv234	428	9.81		Ankyrin repeat family
240c	41	F	fpv234	428	7.01		Ankyrin repeat family
241	**279**	**I**	**fpv236**	**280**	**76.79**		**N1R/p28 family**
242	122	I	fpv237	67	26.83		Hypothetical protein
243	72	F	fpv239	163	31.9	A40R	C-type lectin family
244	**411**	**I**	**fpv240**	**410**	**79.08**		**Ankyrin repeat family**
245	209	I	cnpv313	218	51.14		Ig domain protein
246	628	I	cnpv314/fpv242-243	584	50.94		Ankyrin repeat family
247	192	I	Cnpv309/fpv241	196	29.8		Ankyrin repeat family
248	54	F	cnpv320/fpv017	469	4.26		Ig domain protein
249	667	I	fpv244	688	79.19		
250	75	F	fpv245	463	10.69		Ankyrin repeat family
251a	227	T	cnpv014/fpv017	490	27.09		Ig Domain protein
251b	235	T	cnpv014/fpv017	490	27.96		Ig Domain protein
252	585	I	fpv246	592	85.33		Ankyrin repeat family

253	122	I	fpv247	124	82.26		Efc family
254	149	I	fpv248	151	78.15		N1R/p28 family
255	107	I	fpv249	105	61.4		Hypothetical protein
256	124	I	fpv258	123	68	A40R	C-type lectin family
257	**222**	**I**	**fpv259**	**222**	**91.44**		**Hypothetical protein**
258	66	F	Aasi_1435/ Cnpv021	1585	2.27		Hypothetical protein Aasi_1435 [Candidatus Amoebophilus asiaticus 5a2]
259	**200**	**I**	**fpv260**	**205**		**A40R**	**C-type lectin family**

Bold and Italic: **The 90 core genes conserved in all ChPVs which are involved in essential functions such as replication, transcription and virion assembly. Bold: An additional 88 genes conserved in all avipoxviruses. Gene status is depicted as "I" for intact genes, "F" for fragmented genes, "T" for truncated genes and "a" where the gene is absent from the genome. [α] best BlastP hit (Where the best BlastP hit was a CNPV gene , the homologous fowlpox virus gene is indicated where applicable. Some CNPV genes are not present in FWPV.)**

Table 4.2. Deletions and disruptions in FeP2 relative to FPVUS. Putative functions for the deleted genes have been given where possible

FeP2	Function
Deleted genes	
fpv003	C-type lectin family
fpv004	hypothetical protein
fpv005	Efc family
fpv007	hypothetical protein
fpv008	C-type lectin family
fpv009	hypothetical protein
fpv013	hypothetical protein
fpv027	G-protein coupled receptor family
fpv032	Dnase II
fpv033	α-SNAP
fpv036	hypothetical protein
fpv042	hypothetical protein
fpv045	hypothetical protein
fpv072	β-NGF
fpv073	IL-18 binding protein
fpv115	Ankyrin repeat family
fpv121	CC-chemokine family
fpv122	B22R
fpv123	B22R
fpv124	N1R/p28 family
fpv125	V-type Ig Domain
fpv152	HT-motif
fpv154	hypothetical protein
fpv210	hypothetical protein
fpv220	hypothetical protein
fpv224	Ankyrin repeat family
fpv225	hypothetical protein
fpv238	hypothetical protein
fpv250	US ORF2
fpv251	Serpin
fpv252	hypothetical protein
fpv253	C-type lectin family
fpv254	hypothetical protein
fpv255	C4L/C10L-like family
fpv256	Efc family
fpv257	hypothetical protein
Disrupted genes	
fpv026 fragmented	ankyrin repeat
fpv028 truncated	hypothetical protein
fpv034 fragmented	ankyrin repeat
fpv063 truncated	hypothetical protein
fpv064 fragmented	glutathione peroxidase
fpv072 fragmented	β-NGF
fpv073 truncated	IL-18 binding protein
fpv162.2 Fragmented	ankyrin repeat
fpv177 truncated	hypothetical protein
fpv199 fragmented	V-Type Ig Domain
fpv200 fragmented	V-Type Ig Domain
fpv217 fragmented	hypothetical protein
fpv218 truncated	ankyrin repeat
fpv223 fragmented	ankyrin repeat
fpv228 fragmented	ankyrin repeat
fpv234 fragmented	ankyrin repeat
fpv239 fragmented	C-type lectin
fpv245 fragmented	ankyrin repeat

Table 4.3. Insertions in FeP2 relative to FPVUS. Inserted genes have been listed if they are present in FeP2 in an unexpected site as predicted by genomic synteny with the FPVUS. Where no homology to FPVUS genes is present, the name of the best BlastP hit has been used.

FeP2	Function
Inserted genes	
Fragmented cnpv021	ankyrin repeat
Fragmented cnpv006	hypothetical protein
cnpv320	Ig Domain protein
Fragmented cnpv309	ankyrin repeat
cnpv015	ankyrin repeat
Fragmented Trichomonas vaginalis	ankyrin repeat
fpv244	ankyrin repeat
fpv246	ankyrin repeat
IL-10 (Ficedula albicollis)	IL-10
cnpv028	ankyrin repeat
cnpv037	hypothetical protein
cnpv044	ankyrin repeat
cnpv046	ankyrin repeat
cnpv232	CC-chemokine-like protein
cnpv095	hypothetical protein
Fragmented cnpv098	hypothetical protein
fp03L	vaccinia 03L ortholog
Fragmented cnpv011	ankyrin repeat
Fragmented cnpv004	ankyrin repeat
cnpv012	hypothetical protein
cnpv224	hypothetical protein
cnpv170	thymidilate kinase
Fragmented E. Bacterium	hypothetical
cnpv210	N1R/p28-like protein
cnpv211	hypothetical protein
cnpv212	N1R/p28-like protein
Truncated cnpv279	beta-NGF protein
Fragmented protein (X. tropicalis)	hypothetical
Tanapox 67R	Host Range Protein
Fragmented O.tsutsugamushi str	ankyrin repeat
cnpv301	ankyrin repeat
cnpv313	Ig Domain protein
Fragmented cnpv320	Ig Domain protein
Truncated cnpv014	Ig Domain protein
Fragmented cnpv021	ankyrin repeat

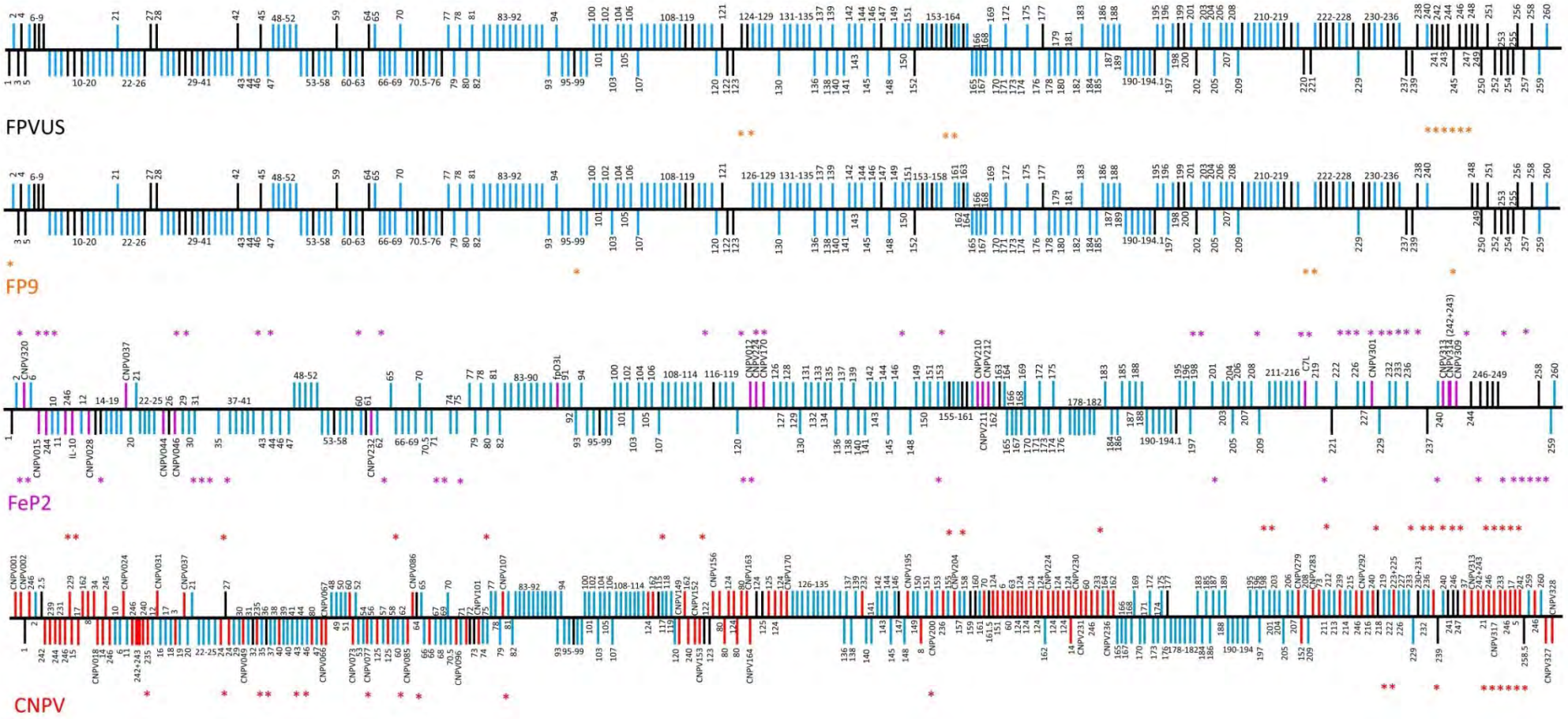


Figure 4.4 Gene content of the 4 avipoxviruses FPVUS, FP9, FeP2 and CNPV. FPVUS is used as the reference genome against which the other avipoxviruses (are compared. For each genome, upward vertical barsrepresent genes transcribed towards the right and downward vertical bars represent genes transcribed towards the left. The genes that are shared amongst all 5 sequences are represented in blue. Black bars represent genes that are shared by at least 2 viruses, but not all. Differences, as compared to FPVUS, are represented in each genome as the same colour as the virus name. Deletions relative to FPVUS are indicated by asterisks.

Table 4.4. A summary of the numbers of intact gene family proteins in FeP2, FPVUS, FP9, CNPV and VACV.

	FeP2	FPVUS	FP9	CNPV	VACV
Ankyrin repeat proteins	26	31	22	51	17
B22R	4	6	5	6	1
N1R/p28	11	10	8	26	?
C4L/C10L	2	3	3	3	3
CC chemokine	4	4	4	5	2
C-type Lectin	4	9	6	11	2
G protein-coupled receptor gene family	2	3	2	4	?
HT Motif	4	6	6	5	-
Ig-like domain protein	4	5	4	9	3
Serpin	4	5	5	5	2
Efc family	1	3	2	2	-
TGF-β	1	1	1	5	-
β-NGF	0	2	2	2	-
interleukin 18 (IL-18)-binding protein	0	1	1	3	1

4.3.7 Gene Translocations and Duplications

Analysis of the FeP2 genome revealed several occurances of gene duplication and translocation relative to FWPV (Table 4.1, Fig. 4.4). In some instances the position of the gene was closer to that observed in CNPV than FWPV. A second copy of the ankyrin repeat family gene, fpv244 orthologue (fep010; cnpv009) is found in the equivalent place in the left hand region of FeP2, between nucleotides 15367-13325.

4.3.8 Disrupted and Deleted Genes

Relative to FPVUS, FeP2 contains 5 truncated FPVUS genes and the fragmented forms of 13 FPVUS orthologues (Table 4.2). FeP2 contains truncated or fragmented remains of ORFs with similarity to CNPV (Table 4.3). Relative to the FPVUS genome, FeP2 has 36 deleted ORFs (Table 4.2) that are absent from any potential coding regions of its genome.

4.3.9 Inserted genes

Relative to FWPV, the FeP2 genome contains several inserted genes (Table 4.3). FeP2 contains an orthologue of a TMPK (fep126, cnpv170; VACV A48R) (Table 4.3) and a putative IL-10 gene (fep014) with limited similarity to the CNPV orthologue (22.05% aa identity) (Fig. 4.5). The amino acid identities between avian IL-10 genes and the CNPV IL-10 orthologue (18.7- 22.7%) is lower than that for the FeP2

orthologue (22.3-28.3%). The FeP2 and CNPV IL-10 genes are most similar to that of a Collared Flycatcher (*Ficedula albicollis*) sharing 28.3% and 22.7% amino acid identity respectively (Fig. 4.5). FWPV does not encode an IL-10 gene [25].

FeP2 encodes a homologue of tanapox virus (TANV) and YLDV 67R which are orthologues of VACV C7L (fep223). Based on MUSCLE alignments of several poxvirus C7L orthologues, TANV and YLDV 67R orthologues share 97.19% amino acid identity. FeP2 shares 26.0% amino acid identity with TANV 67R and 25.4% with YLDV 67R. The FeP2 gene contains putative C7/F8A protein family conserved domains (PFAM accession: pfam03287, E-value: 7.09e-24) which are conserved across all pox C7L proteins.

		1	2	3	4	5	6	7	8	9
CNPV (cnpv018)	1		22.05	18.69	22.73	20.30	22.22	20.20	20.71	20.10
FeP2 (fep014)	2	152		22.28	28.26	27.32	27.17	26.63	26.09	27.07
Human [Homo sapiens]	3	161	143		43.65	41.99	45.30	42.54	44.20	40.33
Collared Flycatcher [Ficedula albicollis]	4	153	132	102		65.54	66.10	61.14	64.57	61.76
Rock Pigeon [Columba livia]	5	157	133	105	61		76.84	74.01	77.97	72.16
Mallard Duck [Anas platyrhynchos]	6	154	134	99	60	41		75.71	79.10	70.06
Turkey [Meleagris gallopavo]	7	158	135	104	68	46	43		92.57	84.57
Chicken [Gallus gallus]	8	157	136	101	62	39	37	13		88.00
Japanese quail [Coturnix japonica]	9	155	132	108	65	49	53	27	21	

Figure 4.5 Pairwise alignment table comparing the amino acid sequences of avipoxvirus Interleukin 10 (IL-10) orthologues with selected avian IL-10 genes. Percentage amino acid identity (top left) and number of differences (bottom left) are depicted.

4.3.10 Comparison of FeP2 to FWPV with respect to attenuation and lineage specific mutations

In order to determine lineage specific mutations, Laidlaw and Skinner (2004) determined the sequence of HP1 (European strain), the virulent parent of FP9, at all loci where FP9 differed from FPVUS (American strain) [27]. Here we compared nucleotide changes in FeP2 at 43 genomic positions influencing the amino acid composition of 25 ORFs of European and American strains as previously identified [27]. In 15 places, FeP2 was the same as HP1 (European lineage) and in 18 places, FeP2 was the same as FPVUS (American lineage). FeP2 differs from both HP1 and FPVUS in 10 places.

4.4 Discussion

The genome sequence of a novel South African APV, FeP2 has been determined and is significantly different from that of CNPV (approx. 63% identity) and FWPV (approx. 85% identity). In OPVs, the internal region of different species share at least 96% identity when compared at the nucleotide level, while different strains from the same species share at least 99% nucleotide sequence identity [311]. These identities have been listed as criteria for establishing poxvirus taxonomy [312]. Therefore, according to the identity between the FeP2 and FPVUS genomes (85% nucleotide identity), as well as the differences in phylogenetics and growth characteristics between these two viruses (chapter 2 and 3), one can conclude that FeP2 is a separate species of APV.

Laidlaw and Skinner (2004) [27] identified geographical lineage-specific mutations which distinguished European FWPVs, FP9 and HP1 from the American strain FPVUS. A comparison of FeP2 with the published FWPV sequences at these sites shows that FeP2 shares lineage-specific mutations with both HP1 and FPVUS. FeP2 is no more closely related to either the American or the European virus, suggesting that FeP2 originates from a common ancestor that diverged relatively recently from a FWPV-like progenitor which was more distantly related to the CNPV-like progenitor. It is important to note that the divergence between APV species is comparable to that between some poxvirus genera and the different species within the OPV genus are highly similar.

FeP2 is most closely related to FWPV. Despite this, relative to FWPV, the FeP2 genome contains several genes that are more closely related to CNPV. FeP2 encodes 16 intact CNPV orthologues (Table 4.3), four of which are not in FWPV, including a TMPK protein (cnpv170, fep126) (Table 4.1 and Table 4.3). The remainder are more similar to CNPV orthologues but are present in FWPV. Rather than being acquired through horizontal gene transfer or recombination, these genes are most probably from a common ancestor. Their existence suggests that FeP2 diverged prior to the present day FWPV, somewhere between the CNPV and FWPV branches. Several of the CNPV-like genes exist as gene fragments (Table 4.3), which is indicative of their gradual loss through progressive mutation, rearrangements and/or deletions over many rounds of virus replication.

There are several instances of gene duplication and translocation in FeP2 relative to FWPV (Fig. 4.4). Most of these involve genes containing ankyrin repeats. Virulent FWPV contains 31 ankyrin repeat containing genes and attenuated FP9 contains 22 of these genes. It is postulated that loss of these genes may be responsible for the attenuation of the virus [25, 27]. FeP2 contains 26 ankyrin repeat-containing genes (Table 4.1; Table 4.4). Thus the reduced numbers of intact ankyrin repeat genes observed in FeP2 compared to FWPV would suggest that this virus is less virulent. On the other hand, the expansion of the number of ankyrin repeat genes in certain APV strains could represent the forming of adaptive genomic accordions, similar to that of the K3L gene in OPV, the formation of which has been described to play an adaptive role in these viruses [313].

The most striking difference between FeP2 and FWPV is a large deletion of ~16kb from the central, usually conserved, region of the genome. ORFs corresponding to fpv121 (cc-chemokine family), fpv122 and fpv123 (VAR B22R family), fpv124 (N1R/p28 family) and fpv125 (V-type Ig domain gene family) are deleted in FeP2 at this site. The existence of gene fragments and insertions on the borders of this deletion, as well as in the equivalent regions in FWPV, suggests that this is a ~~-~~hotspot" of genetic change, the mechanism of which is uncertain.

CC chemokines attract immune cells to sites of infection [314]. MCV encodes a CC chemokine-like protein (MC148R) which antagonises host CC and CXC chemokines [315]. Similarly, APV encode several copies of CC chemokine-like genes which may function as antagonists or agonists of the CC chemokines to modify the host response to viral infection [25][25][25]. The loss of the CC-chemokine orthologue in FeP2 (corresponding to fpv121) may affect the ability of FeP2 to inhibit the action of host CC chemokines. VACV B22R is a member of the serine protease inhibitor family (serpins), which regulate immune responses and cell death by acting as decoys for their target proteinases. VACV B22R (SPI-1) plays a role in the reduction of the host immune response to the virus, and the rabbitpox orthologue of B22R inhibits apoptosis [41, 316]. Studies with VACV have shown that deletion of B22R attenuates VACV and increases its safety without reducing immunogenicity [41]. The deletion of two of these genes in FeP2 may have a similar effect. The FPVUS genes, fpv124 and fpv125, encoding the Rabbit fibroma virus N1R/Ectromelia virus p28 (N1R/p28) gene family protein and a V-type Ig domain family protein respectively are deleted in FP9 as well as in FeP2 [27]. APV immunoglobulin (Ig) domain genes have potential roles in immunomodulation [24] and N1R/p28 genes

are implicated in host range and viral virulence [25]. The five FPVUS genes that are deleted from the central region of FeP2 are all involved in viral host range and virulence, and their loss may indicate that FeP2 is more attenuated than FPVUS. This is supported by the fact that the attenuated FWPV strain, FP9, lacks two of these genes (fpv124 and fpv125) as a result of attenation (passage)-specific mutations [27].

Relative to the FPVUS genome, FeP2 has 36 deleted ORFs (Table 4.2). Most of the genes deleted are members of multi-gene families, the disruption of which has been implicated in the attenuation of poxviruses [27], however there are several other deletions of significant ORFs. Of interest, the orthologue of fpv032 encoding a DNase II has been deleted in FeP2. In FWPV, fpv032 represents the large subunit of cellular DNase II [25, 317] and cellular DNase II is thought to function in DNA catabolism during apoptosis [318, 319]. Additionally, the orthologue of fpv033 encoding one of the two α-SNAP genes present in FWPV, is absent from FeP2. Eukaryotic α-type soluble NSF attachment proteins (α-SNAP) are involved in vesicular transport through the Golgi apparatus and for exocytosis [320, 321]. The fpv033 gene has been shown to be conserved in FWPV strains but non-essential to viral replication and it is thought to be involved in virus-host interactions [322]. Although the fpv033 gene orthologue has been deleted in FeP2, a second α-SNAP-like gene (fep012, fpv011) exists in the genome which exhibits 34% amino acid identity with fpv033.

FWPV contains two genes with homology to cellular β-nerve growth factor (β-NGF) (fpv072 and fpv076). In FeP2, the orthologue of fpv076 is completely deleted and the orthologue of fpv072 (fep074) is fragmented (Table 4.2). An additional β-NGF-like gene is observed in a truncated form in FeP2 (fep213) (Table 4.3). This is most similar to the CNPV gene cnpv279, which exists as a fragment in FWPV [24]. The FeP2 ORF, fep213 is truncated to 67 amino acids by the introduction of a premature stop codon, where the intact cnpv279 orthologue is 169 amino acids. It is unknown whether this protein would be functional in FeP2. β-NGF has proinflammatory properties and is produced by fibroblasts and keratinocytes in response to injury [323-328]. FWPV encoded β-NGF is thought to be involved in promoting survival in infected cells and could have a role in inhibiting antiviral immune responses in the host [25].

FeP2 (fep126) contains an orthologue of TMPK (cnpv170; VACV A48R), which is absent in FWPV [25]. This is the only difference found with respect to the complement of nucleotide metabolism genes. Interestingly, despite being within the conserved central region of the genome, the TMPK gene orthologues occur within a region of FeP2 that is highly variable compared to FWPV. In addition, the CNPV orthologue insertions that occur within this particular variable region in FeP2 occur in different sites across the CNPV genome. The higher than average amino acid identity shared between FeP2 and the CNPV TMPK (81.6%) suggests that this TMPK gene is necessary and conserved in these viruses. CNPV and FeP2 are the only poxvirus species outside of the OPV genus to contain a TMPK homologue. VACV encodes TMPK (A48R) that is 42% identical to human TMPK [329]. TMPK catalyzes an important step in the biosynthesis of (deoxy) thymidine triphosphate and is essential for cell metabolism [330]. The presence of this gene in CNPV and FeP2 may affect cell tropism in these viruses compared to FWPV [24].

FeP2 encodes a putative IL-10 gene. Putative orthologues of IL-10 are also found in ORF virus, BPSV [9], LSDV [187] and YLDV [188]. IL-10 is a cytokine that has both immunostimulatory and immunosuppressive functions [186] and the ORF virus IL-10 orthologue has been shown to be immunomodulatory in function [189, 190, 331]. The IL-10 gene encoded by FeP2 contains two functional domains of the IL-10 superfamily (pfam00726, e-value = 2.40e-14 and smart00188, e value= 2.43e-13) and may also be involved immune evasion.

FeP2 encodes a gene with homology to TANV and YLDV 67R (26.0% amino acid identity) which are orthologues of VACV C7L and not found in FWPV or CNPV. The poxvirus C7L family of host range genes functions by mediating poxvirus host range and antagonising the host defence system [332]. VACV C7L orthologues are found in all OPV and most mammalian poxviruses, with the exception of molluscum contagiosum virus and parapoxviruses [333]. This is the first report of a C7L-like gene in APVs. It is possible that this gene has been retained by FeP2 from the last common ancestor between avian and mammalian poxviruses although homologous recombination with another poxvirus cannot be ruled out. In mammalian cells, C7L has been shown to inhibit apoptosis [276], antagonise the anti-viral effects of type I IFNs and Interferon Regulatory Factor 1 (IRF-1) [334, 335] and can antagonize the dsRNA-activated protein kinase (PKR) pathway by inhibiting the phosphorylation of eIF2a [336]. It has previously been suggested that C7L orthologues are an important adaptation of mammalian poxviruses for replication in mammalian hosts [334]. The

presence of C7L orthologues in FeP2 confounds these previous observations but the function of this gene in this APV remains to be determined. YLDV 67L shows only 28-30% identity to the VACV C7L protein but, despite its limited amino acid identity, has been shown to function equivalently in supporting VACV replication in mammalian hosts [333].

The genome sequence of FeP2 has added greatly to the limited repository of genomic information available for the APV genus. In the comparison of FeP2 to existing sequences, FWPV and CNPV, we have established insights into African APV evolution. Although FeP2 is more closely related to FWPV, the presence of whole or disrupted genes with similarity to CNPV genes that are absent in FWPV, suggests that FeP2 originates from the common ancestor of CNPV and FWPV. The presence of an intact gene in CNPV and FeP2 where the FWPV counterpart is fragmented into two ORFs further supports this theory as fragmented genes represent the gradual loss of genetic information during the process of virus evolution. Additional sequences of APV genomes would help to define APV evolution as a whole.

The ongoing search for an ideal vaccine vector makes this work relevant. Future work could focus on how this APV differs from the well characterized FWPV and CNPV with respect to immune activation and foreign gene expression. Microarray analysis which looks at gene expression changes induced by the different viruses in a model system will be beneficial to determine how differences in APV genomes may influence APV recognition and behaviour *in vitro*, or *in vivo*.

Chapter 5
Assessment of early host responses induced by different poxviruses in mice

5.1 Introduction

Many different poxviruses are available as potential vaccine vectors. In addition to being highly immunogenic, poxviruses such as MVA, LSDV and APVs are host restricted and/or attenuated. MVA, an Orthopoxvirus, was derived from vaccinia virus Ankara, which was passaged over 570 times in chicken embryo fibroblasts resulting in attenuation and severe restriction of host range, as a result of numerous genomic deletions rendering it unable to replicate in most mammalian cells [281, 337]. LSDV is a capripoxvirus, permissive only to ruminants [338]. APVs are host restricted to cells of avian origin [45, 275].

MVA is a successful vaccine vector against a variety of pathogens and malignancies [70, 339]. LSDV has been proposed as a novel vaccine vector for different human and animal pathogens, including HIV and rabies [338]. APVs have been used effectively as vaccine vectors against a number of mammalian and human pathogens [76]. Poxvirus vaccine vectors have been shown to be an important component of heterologous prime-boost strategies to induce HIV responses in clinical trials including planned efficacy trials [340, 341].

Recent studies of different vaccines including yellow fever vaccine and influenza vaccine have revealed early host gene signatures which are capable of predicting the subsequent vaccine-induced adaptive immune response [342-345]. Host-range restricted poxviruses have been shown to successfully activate the host immune system [85, 86] and evidence exists to indicate that each virus does this in a different way, with an accompanying different pattern of gene expression [87-91]. The poxviruses ALVAC (based on CNPV), MVA and NYVAC (a vaccinia virus with specific deletions) produce distinct innate immune profiles, characterised by different induction of pro-inflammatory and antiviral cytokines and chemokines in both rhesus monkeys and human PBMCs [93].

It has been shown that in non-permissive cells, FWPV proceeds further into the poxvirus life cycle than CNPV [271], and that heterologous HIV gag/pol and env genes are more efficiently expressed by FWPV than CNPV in vitro due to longer transgene expression and more balanced cytokine induction [346]. However, the only successful HIV-1 vaccine clinical trial to date (31.2% protection from HIV-1 infection) has been the Thai RV144 trial involving priming with a CNPV (ALVAC) vector expressing HIV-1 gp120/Gag-Pro and boosting with a recombinant

glycoprotein 120 subunit, AIDSVAX RW [60]. More head to head comparisons of poxvirus-vectored vaccines would help to establish which candidate would be suitable to achieve the desired vaccine-induced response to achieve protection against pathogens and cancers.

Innate immunity is critical for directing the adaptive immune response to antigen and influences the magnitude and quality of the long-lived, protective immune responses to pathogens or vaccines [347]. The identification of innate immune signatures that can predict the consequent adaptive immune response is beneficial in the development and rational design of new vaccine candidates. Chemokines induce the migration of immune effector cells to the site of infection. IL-8 (CXCL8) induction causes the recruitment of neutrophils, whereas MIP-1α and MIP-1β recruit NK cells, macrophages and immature DCs. IP-10 (CXCL10) causes the migration of activated T cells into tissue [348]. Clearly the innate immune response is important in the induction of an adaptive immune response. Systems biology evaluates the complex interactions within a biological system, and is an approach which can be enormously valuable in studying the interplay between virus (or vaccine) and host. Application of the systems biology approach to vaccine development (~~-s~~ystems vaccinology") and establishment of innate immune signatures has proven useful in predicting the immunogenicity of the highly effective yellow fever vaccine (YF-17D) [342], seasonal influenza vaccines [343] and the immunogenic but inefficacious Merck Ad5/HIV vaccine [344]. Post hoc systems analysis of the Merck Ad5/HIV vaccine indicated both that innate immune reponses are able to predict immunogenicity of the vaccine, and that the existence of pre-exisiting neutralizing antibodies to Ad5 attenuate the innate immune response to MRKAd5/HIV, suggesting that the enhanced HIV acquisition in pre-immune individuals in the Step trial may be a result of poor innate activation rather than increased immune activation as previously hypothesized [344]. A better understanding of the mechanisms underlying the optimal innate immune responses would aid rational vaccine development.

Type I IFNs (IFNα/β) are expressed rapidly in response to viral infection. Type I IFNs in turn activate many ISGs which exert various antiviral effector functions. The effect of type I IFNs on poxvirus-induced immune responses is complex and poorly understood. Co-administration of IFN-α with VACV resulted in a decrease in cell mediated immunity whereas IFN-α enhanced antigen-specific T-cell proliferation and cytotoxic T lymphocyte responses when co-administered with FWPV [110]. Conversely, type I IFN is not necessary for the induction of adaptive immunity to

FWPV-encoded antigen [111]. A balance is necessary for vaccination purposes, where enough type I IFNs are produced to activate the immune system, yet not enough to inhibit viral DNA replication and gene expression before antigen presentation can occur [96]. This is corroborated by Johnson et al (2012) [349], who compared rAd5, rAd28 and rAd35. Here they showed that the specific IFN-α induction by rAd28 and rAd35 significantly lowered the immunogenicity of these vectors compared to rAd5 which did not induce IFN-α expression [349]. The effect of type I IFN responses on different vaccines requires delineation of innate immune signatures and how they determine subsequent adaptive responses.

Microarray analyses performed *in vitro* have been used to investigate the effects of VACV [90], MVA [87] and NYVAC [89] infection on HeLa cell gene expression. Gene expression profiles in human monocyte derived dendritic cells (MDDCs) have also been generated with MVA, NYVAC [88] and ALVAC [91]. Furthermore, a comparison of the closely related VACV-derived vectors NYVAC and MVA revealed significant differences in antigen production and host gene dysregulation in cell culture [276]. Consequently it was speculated that genetically diverse poxvirus strains would induce significant differences in host gene expression. The interaction of poxviruses with the host is not just dependent on the actual cell infected by the virus but also on the factors secreted by those infected cells and their effects on the surrounding cells. Although *in vitro* expression studies have provided useful information, gene expression profiles performed in cell culture may not accurately reflect the changes in the system that occurs as a result of infection *in vivo.* A recent study in Rhesus Macaques has shown that the CNPV-based vector, ALVAC, induced distinct cytokine and chemokine levels compared to the vaccinia virus-based vectors MVA and NYVAC and that multiple subsets of PBMCs are likely to contribute to the overall response to different poxviruses [93].

Here we investigate and compare the effects of the capripoxvirus LSDV, the orthopoxvirus MVA, and the four APVs; CNPV, FWPV, FeP2 and PEPV on host gene expression profiles in the spleens of BALB/c mice. The spleen is one of the major peripheral lymphoid organs and functions to collect antigen from the blood and present it to migratory lymphocytes, thereby playing a role in the induction of the adaptive immune response [350]. Comparative analysis of the gene expression profiles in mouse spleens by microarray will yield valuable information on the ability of different host restricted poxviruses to alter mammalian cell pathways on a whole organism level. This has relevance for both human and veterinary vaccines.

5.2 Materials and methods

5.2.1 Viruses

FeP2 was from a Feral Pigeon (*Columba livia*), isolated as described in chapter 2, section 2.2.1. PEPV seedstock was kindly obtained from Olivia Carulei a fellow PhD student at University of Cape Town, originally isolated by D. Kow and Prof. K Dumbell [71]. MVA and wild type CNPV were obtained from Prof. K. Dumbell's collection housed at the University of Cape Town and were originally from Prof. A. Mayr (Veterinary Faculty, University of Munich, Munich, Germany). The FWPV vaccine is DCEP 25 modified strain (Sanofi Merial, Duluth, GA, USA) obtained from a licensed veterinarian and LSDV is a vaccine strain, Herbivac® (Ceva), manufactured by Deltamune (Pretoria, South Africa).

Virus isolates were grown and titrated on the CAMs of embryonated 10-11 day old (MVA, CNPV, FWPV) or 7 day old (LSDV) SPF White Leghorn chicken eggs, which were obtained from Avifarms (Pty) Ltd (Lyttelton, South Africa), using a method described previously (chapter 2, section 2.2.2). Titrations were performed on CAMs for APVs and MVA. Titration of LSDV was performed by immunostaining on Madin Darby bovine kidney (MDBK) cells by Dr. Z. Ginbot.

5.2.2 Virus infection of mice

Mouse work was performed by Rodney Lucas in the University of Cape Town, Health Sciences Faculty Research Animal Facility. Seven week old naive female BALB/c mice were randomly divided into groups of three and each mouse was inoculated intravenously (i.v.) with 10^5 pfu/100ul poxvirus, diluted in PBS or mock infected with PBS alone or egg extract (100µl). The egg extract was made from uninfected CAMs, following the same extraction and purification procedure as the virus samples above. We compared the gene expression profiles of the groups of mice that were mock-infected with egg extract and PBS. For each different virus, three groups of three mice each were inoculated. At 24 hours post infection, the mice were sacrificed by cervical dislocation without anaesthesia and the spleens were harvested and placed in RNAlater (Qiagen, Venlo, Limburg, NL).

5.2.3 RNA extraction

Mouse spleens were removed from RNAlater and the three spleens in each group were pooled and homogenized thoroughly, using a TissueRuptor (Qiagen), in TRIzol® reagent (Life Technologies, Carlsbad, CA, USA). Total RNA was isolated using TRIzol® Plus RNA Purification Kit (Life Technologies, Carlsbad, CA, USA) with On-column PureLink® DNase treatment according to the manufacturer's instructions. RNA was resuspended in RNase free water and quality checked using the Nanodrop ND1000 (Thermoscientific, Waltham, MA, USA) and the Agilent Bioanalyzer Nano Assay (Agilent, Santa Clara, CA, USA). Olivia Carulei assisted with the RNA extractions.

5.2.4 Microarray and data analysis

mRNA hybridization was performed by IMGM Laboratories GmbH (Martinsried, DE) with the Affymetrix GeneChip Mouse Gene 2.0 ST array (Affymetrix, Santa Clara, CA, USA). Data analysis was performed in R [351], using packages from the Bioconductor suite (http://www.bioconductor.org), and CRAN (http://cran.rproject.org). All R code is available in appendix 3. Dr Armin Deffur provided bioinformatics support and input. Probe level data from .CEL files was normalised using the Robust Multi-array Averaging (RMA) method [352] obtained as part of the ~~a~~ffy" package [353] from Bioconductor, resulting in $\log_2$ transformed values. Boxplots, scatterplots and histogram outputs of the normalised data were obtained and checked for consistency (not shown). Data was annotated using the Mouse Gene ST 2.0 annotation data package from Bioconductor. Non-specific filtering was performed using the Genefilter package [354]. This step included an intensity filter which filtered the data set such that the intensity of each gene should be $>\log_2$ (100) in at least 20% of the samples. Secondly, a variance filter was applied such that the interquartile range of $\log_2$-intensities should be at least 0.5.

Differential gene expression was determined using a linear model approach using the R package, Limma [355]. A heatmap was made using heatmap.2 from the CRAN package gplots [356], and depicted the unsupervised hierarchical clustering based on the genes with p-value < 0.05 and $\log_2$ fold change (FC) above or below cutoff (±1). Venn diagrams were made using Venny [357] available at http://bioinfogp.cnb.csic.es/tools/venny/index.html. Gene ontology (GO) enrichment was performed using the R package clusterProfiler [358]. For gene ontology

analysis, all genes with an adjusted p-value of ≤ 0.05, irrespective of fold change, were used to ensure that no information was lost by the arbitrary designation of a fold change cut off. This was done to show the full effect on pathways by the different viruses. Functional analysis was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 web-based tools (www.david.-abcc.-ncifcrf.-gov/-tools.-jsp). Quantitative real time PCR (RT-PCR) was performed on selected genes to validate microarray findings (data not shown).

Histone transcripts

Previous studies have found that increased detection of histone genes by poxvirus infection is described as an experimental artefact due to the de novo polyadenylation of transcripts by the viral poly-A polymerase [92, 359, 360].

5.3 Results

5.3.1 Comparison of the host responses to different poxviruses

We compared the differential host gene expression induced by six host-restricted poxviruses, MVA, LSDV, FWPV, CNPV, FeP2 and PEPV, in the spleens of BALB/c mice at 24h post infection. Transcripts with an adjusted p-value < 0.05 were described as up regulated if they had a $\log_2$ FC of ≥1, or down regulated if they had a $\log_2$ FC of ≤-1. A summary of the number of up and down regulated genes is given in Table 5.1. Full gene lists are given in appendix 4, Tables a1 and a2. Several histone transcripts (39 in total) were down regulated in response to virus infection and, because any interpretation of these transcripts would be speculative, these have been excluded from further analysis.

Unsupervised hierarchical clustering based on the genes with p-value < 0.05 and $\log_2$ FC above or below cutoff (>1, <-1) showed that each virus induced a unique overall response (Fig. 5.1). Venn diagrams highlight the number of differences and similarities in the up- and down regulated genes between the viruses (Fig. 5.2). Each virus modulated a different number of genes, with varying numbers of genes in common (Fig. 5.2). Figures 5.2 A and B show the differences in up- (A) and (B) down regulated transcripts between FWPV, CNPV, MVA and LSDV respectively. Similarly, figures 5.2C and D represent the differences in gene expression between the four APVs analysed. When only the APVs were compared FeP2 up regulated 17 genes in common with all four APVs, two in common with CNPV and one in

common with PEPV. PEPV up regulated 43 genes in common with CNPV and 7 in common with FWPV. FWPV up regulated 166 unique genes and 198 in common with CNPV. CNPV up regulated 19 unique genes. There were only 3 genes down regulated in all four APVs tested. FeP2 had no other genes down regulated. PEPV shared 15 down regulated genes with CNPV and FWPV, and one with CNPV only. FWPV had 28 unique down regulated genes and 16 in common with just CNPV. CNPV had 12 unique genes down regulated.

The Venn diagrams comparing FWPV, CNPV, MVA and LSDV indicate that the majority of up regulated genes are shared amongst these 4 viruses (Fig. 5.2). The down regulated genes however, appear largely unique, especially for LSDV and MVA (Fig. 5.2). FWPV and CNPV down regulate a smaller number of genes in comparison to LSDV and MVA. Comparison of APV-induced up- and down regulated genes shows that FeP2 and PEPV induce significantly less change in host gene expression than FWPV and CNPV (Fig. 5.2). FeP2 induced the lowest response (Fig. 5.2, Table 5.1). For all six viruses, more genes were up regulated than down regulated (Table 5.1).

The differences in the enrichment of genes associated with specific GO terms are displayed in Figure 5.3. This analysis indicates that while PEPV, CNPV, FWPV, MVA and LSDV enrich several common GO terms (eg: biological process), FeP2 fails to do so. FeP2 however up regulates genes that are associated with GO terms that are not enriched in the other 5 viruses, including several GO terms related with development, regulation of response to stimulus and defence response (Fig. 5.3). Although five of the viruses up regulated genes associated with the same GO terms each virus was associated with a unique profile.

Table 5.1. Summary of the number of significantly up- and down regulated transcripts with adjusted p-value < 0.05. Genes are described as up regulated if they had a fold change of ≥2, or down regulated if they had a fold change of ≤-2. These included genes that are not annotated and therefore do not have an Entrez ID. The numbers of genes without annotation are indicated in brackets.

	Up regulated FC>2	Down regulated FC<-2
MVA	299 (42NA)	177 (86NA)
LSDV	463 (111NA)	85 (11NA)
FWPV	433 (101NA)	62 (28NA)
CNPV	280 (31NA)	47 (11NA)
FeP2	20 (1NA)	3 (0NA)
PEPV	68 (6NA)	19 (2NA)

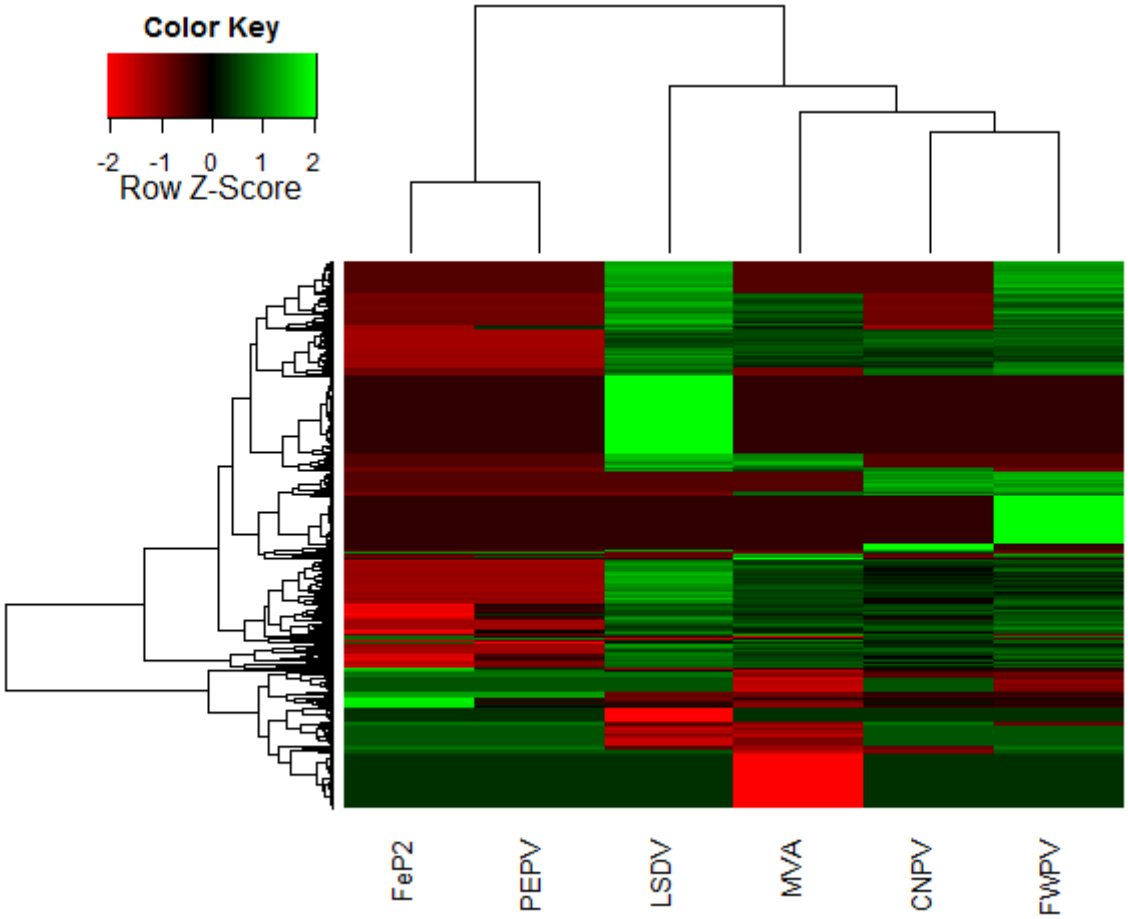


Figure 5.1. Heatmap comparing the differential expression induced in mouse spleens in response to FeP2, PEPV, LSDV, MVA, CNPV and FWPV. Only genes (with p-value<0.05) with $\log_2$ fold change induction above or below the cutoff (±1) as compared to the mock infected control are shown. Unsupervised hierarchical clustering of the samples is represented by dendograms. Clustering analysis and heatmap was performed in the R package, gplots (Warnes, 2009).

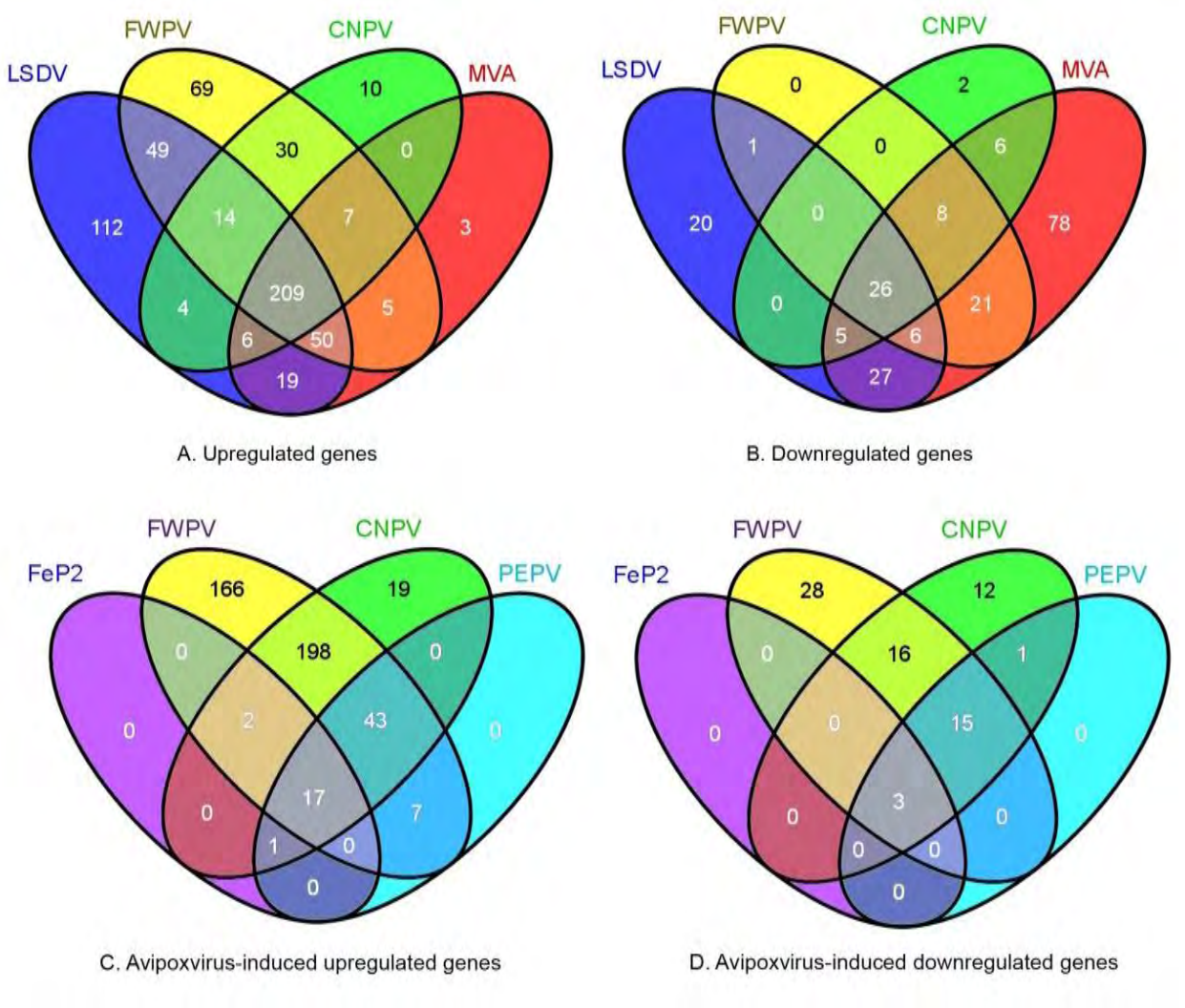


Figure 5.2. Venn diagrams showing the overlap between the differentially up regulated (A) and down regulated (B) transcripts induced by CNPV, FWPV, MVA and LSDV and the up regulated (C) and down regulated (D) transcripts induced by the four avipoxviruses. For each diagram, the circles represent the number of differently expressed transcripts regulated by each virus (p value ≤ 0.05, $\log_2$ fold change of ≥ ±1). The numbers in the intersections of each circle represents the number of transcripts common to the respective virus/es.

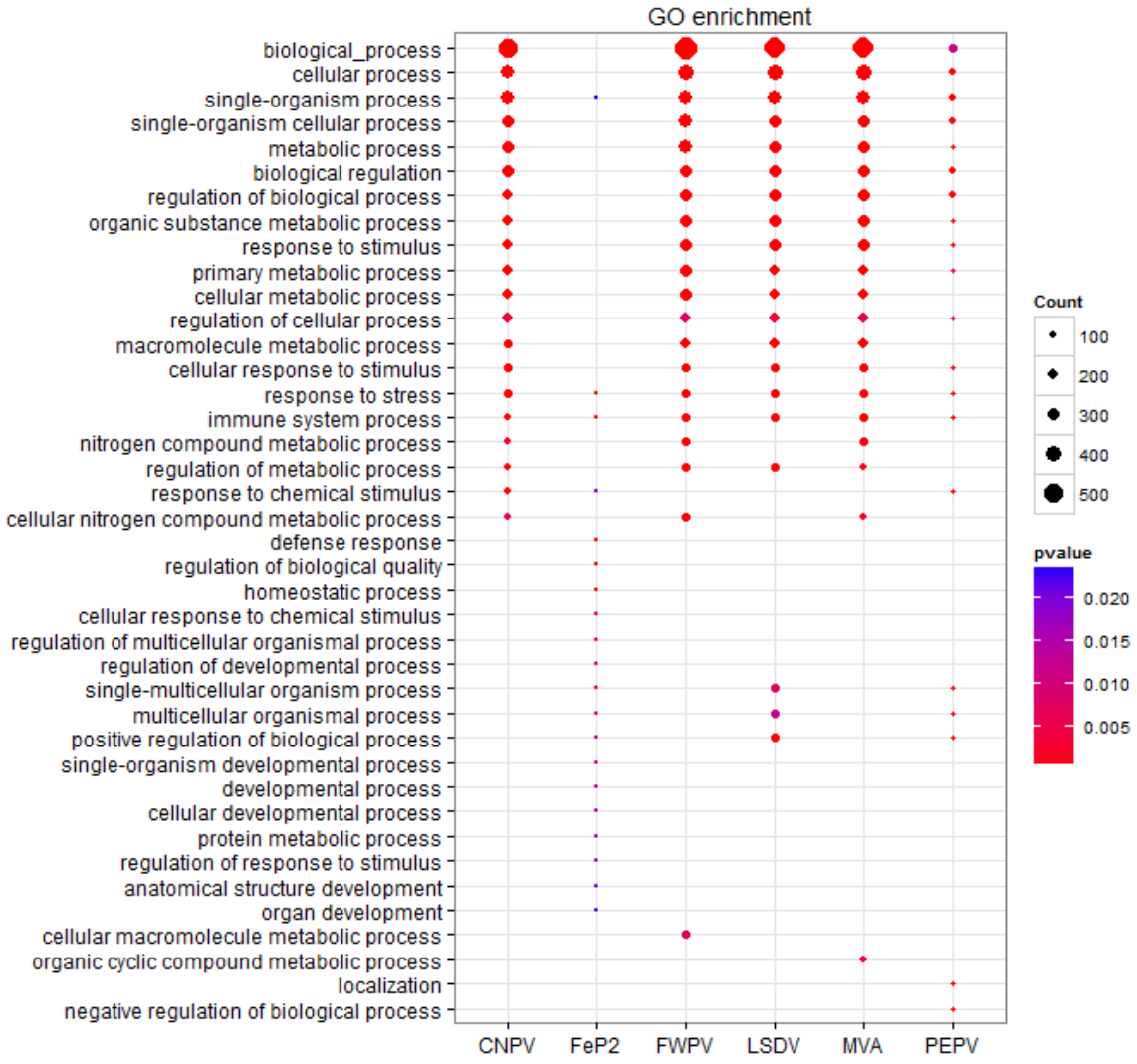


Figure 5.3. Biological theme comparison comparing the significantly enriched (p-value≤0.05) Gene Ontology (GO) terms in CNPV, FeP2, FWPV, LSDV, MVA and PEPV. The above graph is organised by the number of genes (indicated by the size of the dot) assigned to each GO term and by the significance of the association (p-value) (indicated by the colour of the dot).

5.3.2 Immunity and host defence response-related genes

Selected up regulated genes involved in the immune response are listed in Table 5.2. (Full list of up regulated genes is given in appendix 4). Seventeen of these genes are uniquely up regulated by LSDV. RIG-I (Ddx58) senses viral nucleic acid [361], Cebpb is important for macrophage function [362] and control of inflammatory responses [363], Tap1 and Tap2 genes are involved in antigen presentation to MHC class 1 molecules [364], Ifitm3 and Ifi203 are ISGs, c-Myc and Mif are transcription factors and Adar is an RNA editing enzyme. There are 8 genes induced by CNPV, FWPV and MVA, which are not up regulated in LSDV-infected mice. Two of these include the cytidine deaminase, Apobec1, which can edit viral nucleic acid and can thereby limit viral replication [365], and caspase 1 (Casp1), which is associated with pyroptosis (Table 5.2).

Twenty six genes involved in the host immune/defence response were up regulated only in APV -infected mouse spleens (Table 5.2). The only APV - specific gene that was up regulated by all four APVs was the macrophage receptor with collagenous structure (Marco) gene which has been shown to suppress early inflammatory responses to virus infection [366]. There were, however, 9 additional genes which were up regulated by both CNPV and FWPV that were not induced by the other viruses. These included the chemokine Ccl6 which promotes immune cell activation and recruitment [367] and the immunoglobulin heavy chain genes, Ighg (IgG) and Ighg3 (IgG3) (Table 5.2). Amongst these 26 APV-specific genes, 14 were exclusively up regulated by FWPV. The Nod-like receptor, NLR, Nod1, which has been shown to be augmented in response to virus-induced production of type I IFNs [368] was exclusively up regulated by FWPV. Two genes were uniquely up regulated in CNPV-infected mice including the immunoglobulin heavy chain gene, Ighm (IgM) and lymphocyte antigen 96 (Ly96) (Table 5.2).

Four genes were up regulated by LSDV and MVA that were not induced by the APVs in mice (Table 5.2), namely the Moloney Leukemia Virus 10 (Mov10) gene, hematopoietic SH2 domain containing protein (Hsh2d), poly (ADP-ribose) polymerase family, member 11 (Parp11) and schlafen 8 (Slfn8). No genes were uniquely up regulated in response to MVA infection (Table 5.2). PePV and FeP2 induced very little immune activation according to this microarray analysis.

Table 5.2. Selection of up regulated genes in mouse spleens in response to CNPV, FeP2, FWPV, LSDV, MVA and PEPV. Differences in Log_2 Fold Changes (between each virus and the control) are depicted.

Symbol	Name	Entrez	MVA	LSDV	CNPV	FWPV	PEPV	FeP2
GENES INDUCED BY LSDV ALONE.								
Oas1b	*2'-5' oligoadenylate synthetase 1B*	*23961*	-	*1.6*	-	-	-	-
Adar	*adenosine deaminase, RNA-specific*	*56417*	-	*1*	-	-	-	-
Cebpb	*CCAAT/enhancer binding protein (C/EBP), beta*	*12608*	-	*1*	-	-	-	-
Ddx58 (RIG-1)	*DEAD (Asp-Glu-Ala-Asp) box polypeptide 58*	*230073*	-	*1.3*	-	-	-	-
Grn	*granulin*	*14824*	-	*1.1*	-	-	-	-
Gvin1	*GTPase, very large interferon inducible 1*	*74558*	-	*1.5*	-	-	-	-
Gm17757	*GTPase, very large interferon inducible 1 pseudogene*	*100417829*	-	*1.5*	-	-	-	-
H2-T24	*histocompatibility 2, T region locus 24*	*15042*	-	*1.2*	-	-	-	-
Ifi203	*interferon activated gene 203*	*15950*	-	*1.1*	-	-	-	-
Ifitm3	*interferon induced transmembrane protein 3*	*66141*	-	*1.4*	-	-	-	-
Ifi27l2a	*interferon, alpha-inducible protein 27 like 2A*	*76933*	-	*1.9*	-	-	-	-
Ly6i	*lymphocyte antigen 6 complex, locus I*	*57248*	-	*1*	-	-	-	-
Mif	*macrophage migration inhibitory factor*	*17319*	-	*1.1*	-	-	-	-
Myc	*myelocytomatosis oncogene*	*17869*	-	*1.1*	-	-	-	-
Nlrc5	*NLR family, CARD domain containing 5*	*434341*	-	*1.8*	-	-	-	-
Slfn2	*schlafen 2*	*20556*	-	*1.1*	-	-	-	-
Stat1	signal transducer and activator of transcription 1	20846	-	1.4	-	-	-	-
Tap1	*transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)*	*21354*	-	*1.1*	-	-	-	-
Tap2	*transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)*	*21355*	-	*1*	-	-	-	-
Trim25	*tripartite motif-containing 25*	*217069*	-	*1*	-	-	-	-
Trim34b	*tripartite motif-containing 34B*	*434218*	-	*1.1*	-	-	-	-
GENES INDUCED BY CNPV, FWPV AND MVA ONLY, AND NOT LSDV.								
Casp1	*caspase 1*	*12362*	*1.1*	-	*1.2*	*1.1*	-	-
Clec4a2	*C-type lectin domain family 4, member a2*	*26888*	*1.2*	-	*1.5*	*1.1*	-	-
Ifi205	*interferon activated gene 205*	*226695*	*1.4*	-	*1.6*	*1.8*	-	-
Prdx1	*peroxiredoxin 1*	*18477*	*1*	-	*1*	*1.2*	-	-
Pnpt1	*polyribonucleotide nucleotidyltransferase 1*	*71701*	*1.1*	-	*1.1*	*1.4*	-	-
Scimp	*SLP adaptor and CSK interacting membrane protein*	*327957*	*1.2*	-	*1.2*	*1.5*	-	-
GENES INDUCED BY AVIPOXVIRUSES ONLY (CNPV, FWPV, FEP2 AND/ OR PEPV)								
Anxa1	**annexin A1**	**16952**	-	-	**1.6**	**1.8**	-	-
Apobec1	**apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1**	**11810**	-	-	**1.2**	**1.2**	-	-
Ccl6	**chemokine (C-C motif) ligand 6**	**20305**	-	-	**1.3**	**1.3**	-	-
Ear2	**eosinophil-associated, ribonuclease A family, member 2**	**13587**	-	-	**1.3**	**1.5**	-	-
Hsbp1	**heat shock factor binding protein 1**	**68196**	-	-	**1.2**	**1.2**	-	-
Ighg	**Immunoglobulin heavy chain (gamma polypeptide)**	**380794**	-	-	**1.5**	**1.6**	-	-
Ighg3	**Immunoglobulin heavy constant gamma 3**	**380795**	-	-	**1.3**	**1.2**	-	-
Lilrb3	**leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3**	**18733**	-	-	**1.1**	**1**	-	-
Marco	**macrophage receptor with collagenous structure**	**17167**	-	-	**1**	-	**1.5**	**1.5**
Pf4	**platelet factor 4**	**56744**	-	-	**1.2**	**1.1**	-	-
Pram1	**PML-RAR alpha-regulated adaptor molecule 1**	**378460**	-	-	**1.1**	**1.1**	-	-
Psma1	**proteasome (prosome, macropain) subunit, alpha type 1**	**26440**	-	-	**1.1**	**1.3**	-	-
GENES INDUCED BY FWPV ONLY.								
Aif1	***allograft inflammatory factor 1***	***11629***	-	-	-	***1.1***	-	-
Anxa2	***annexin A2***	***12306***	-	-	-	***1.1***	-	-
Ddx18	***DEAD (Asp-Glu-Ala-Asp) box polypeptide 18***	***66942***	-	-	-	***1.1***	-	-
Dcn	***decorin***	***13179***	-	-	-	***1.5***	-	-
Fgl2	***fibrinogen-like protein 2***	***14190***	-	-	-	***1.2***	-	-
Gsdmd	***gasdermin D***	***69146***	-	-	-	***1.2***	-	-
Myd88	***myeloid differentiation primary response gene 88***	***17874***	-	-	-	***1.2***	-	-
Nos2	***nitric oxide synthase 2, inducible***	***18126***	-	-	-	***1.1***	-	-
Nod1	***nucleotide-binding oligomerization domain containing 1***	***107607***	-	-	-	***1***	-	-
Pdcd5	***programmed cell death 5***	***56330***	-	-	-	***1.2***	-	-
Psmc6	***proteasome (prosome, macropain) 26S subunit, ATPase, 6***	***67089***	-	-	-	***1***	-	-
Prmt1	***protein arginine N-methyltransferase 1***	***15469***	-	-	-	***1***	-	-
Serpinb6b	***serine (or cysteine) peptidase inhibitor, clade B, member 6b***	***20708***	-	-	-	***1.2***	-	-
GENES INDUCED BY CNPV ONLY.								
Ctsl	**cathepsin L**	**13039**	-	-	**1.1**	-	-	-
Ighm	**immunoglobulin heavy constant mu**	**16019**	-	-	**1.1**	-	-	-

Ly96	**lymphocyte antigen 96**	**17087**	-	-	**1.2**	-	-	-
Pomp	**proteasome maturation protein**	**66537**	-	-	**1**	-	-	-
GENES INDUCED BY LSDV AND MVA, BUT NOT BY THE AVIPOXVIRUSES								
Hsh2d	hematopoietic SH2 domain containing	209488	1.1	1.4	-	-	-	-
Mov10	Moloney leukemia virus 10	17454	1.1	1.5	-	-	-	-
Parp11	poly (ADP-ribose) polymerase family, member 11	101187	1	1.4	-	-	-	-
Slfn8	schlafen 8	276950	1.2	1.5	-	-	-	-
OTHER								
Oas1a	2'-5' oligoadenylate synthetase 1A	246730	1.5	2.7	1.6	1.4	-	-
Oas1g	2'-5' oligoadenylate synthetase 1G	23960	2.3	3.9	2.5	2.3	-	-
Oas2	2'-5' oligoadenylate synthetase 2	246728	2.1	3.4	2	1.6	-	-
Oas3	2'-5' oligoadenylate synthetase 3	246727	1.1	2.6	1.3	1	-	-
Oasl1	2'-5' oligoadenylate synthetase-like 1	231655	2.6	3.5	2.4	2.5	-	-
Oasl2	2'-5' oligoadenylate synthetase-like 2	23962	2	3.4	2	2	-	-
Amica1	adhesion molecule, interacts with CXADR antigen 1	270152	-	1.1	-	1.1	-	-
Angptl4	angiopoietin-like 4	57875	1.1	1.5	-	1.6	1.1	-
Asb13	ankyrin repeat and SOCS box-containing 13	142688	1.2	1	1.1	1.3	-	-
Anxa4	annexin A4	11746	1.8	1.9	1.7	2.1	1.1	-
Apol9b	apolipoprotein L 9b	71898	2.3	2.7	2.6	2.4	-	-
Bst2	bone marrow stromal cell antigen 2	69550	2.3	3	2.1	2.2	-	-
Casp4	caspase 4, apoptosis-related cysteine peptidase	12363	1.9	1.6	1.9	2	-	-
Ctsc	cathepsin C	13032	1.1	1.1	-	1.1	-	-
Cd274	CD274 antigen	60533	1.9	2.3	1.6	2.1	1.2	-
Cd5l	CD5 antigen-like	11801	1.2	1.5	1.4	1.1	-	-
Cd69	CD69 antigen	12515	1.8	1.8	1.5	1.7	-	-
Ccl2 (MCP1)	chemokine (C-C motif) ligand 2	20296	3.5	3.3	2.9	3.3	2.8	-
Ccl3 (MIP-1α)	chemokine (C-C motif) ligand 3	20302	2	2.1	2.3	2	1.4	-
Ccl7	chemokine (C-C motif) ligand 7	20306	3	2.9	2.6	2.9	2.7	1.5
Ccr5	chemokine (C-C motif) receptor 5	12774	1.1	1.5	1.3	1.3	-	-
Ccrl2	chemokine (C-C motif) receptor-like 2	54199	-	1.5	1.2	1.4	-	-
Cxcl10 (IP-10)	chemokine (C-X-C motif) ligand 10	15945	2.7	3	2.2	2.8	1.7	-
Cxcl11 (I-TAC)	chemokine (C-X-C motif) ligand 11	56066	4.5	4.4	3.4	4.3	1.5	-
Cxcl9 (MIG)	chemokine (C-X-C motif) ligand 9	17329	2.1	2.3	-	1.8	-	-
Chi3l3	chitinase 3-like 3	12655	-	1.2	1.4	1.6	-	-
Csf2rb2	colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage)	12984	-	1.5	-	1.7	-	-
C1qa	complement component 1, q subcomponent, alpha polypeptide	12259	-	1.1	1	-	-	-
C2	complement component 2 (within H-2S)	12263	1.5	1.8	1.7	1.6	1	-
Cfb	complement factor B	14962	2.2	2.6	1.6	2	1	-
Cdkn1a (P21)	cyclin-dependent kinase inhibitor 1A	12575	1.7	2	1.5	2	1.2	-
Cstb	cystatin B	13014	1.4	1	1.5	1.5	-	-
Cst7	cystatin F (leukocystatin)	13011	1.2	1.4	1.1	1.4	-	-
Cmpk2	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	22169	1.3	2	1.3	1.3	-	-
Cycs	cytochrome c, somatic	13063	-	1.1	-	1.1	-	-
Ctla2a	cytotoxic T lymphocyte-associated protein 2 alpha	13024	1.1	1	1.1	1.3	-	1.4
Ddx60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	234311	1.7	2.6	1.5	1.4	-	-
Dhx58 (LGP2)	DEXH (Asp-Glu-X-His) box polypeptide 58	80861	1.7	2.5	1.6	1.5	-	-
Dram1	DNA-damage regulated autophagy modulator 1	71712	1.2	1.4	-	1.3	-	-
Ddit4	DNA-damage-inducible transcript 4	74747	1.2	1.2	1.8	1.3	1.4	-
Eif2ak2 (PKR)	eukaryotic translation initiation factor 2-alpha kinase 2	19106	1.1	2.1	1.1	1.1	-	-
Daxx	Fas death domain-associated protein	13163	2	2.3	1.7	1.9	-	-
Fcgr1	Fc receptor, IgG, high affinity I	14129	2.4	2.6	2.4	2.6	1.4	-
Fcgr4	Fc receptor, IgG, low affinity IV	246256	2.5	3.6	2.8	2.9	1.8	-
Fpr1	formyl peptide receptor 1	14293	1.3	1.1	1.4	1.4	-	-
Fpr2	formyl peptide receptor 2	14289	1.8	1.5	1.7	1.9	-	-
Glipr2	GLI pathogenesis-related 2	384009	1.4	1.5	1.1	1.5	-	-
Gp49a	glycoprotein 49 A	14727	2.6	2.4	2.7	2.7	1.9	2
Gca	grancalcin	227960	1.2	1.3	1.4	1.6	-	-
Gzma	granzyme A	14938	1.8	1.8	2.2	2	-	-
Gzmb	granzyme B	14939	3.7	4.7	4.1	4.2	2.4	-

Gadd45b	growth arrest and DNA-damage-inducible 45 beta	17873	1.3	1.5	1.2	1.5	-	-
Gbp1	guanylate binding protein 1	14468	2.1	2.7	1.6	2.5	-	-
Gbp11	guanylate binding protein 11	634650	3.9	4.5	2.9	4.2	1.6	-
Gbp2	guanylate binding protein 2	14469	2.3	2.8	1.5	2.7	-	-
Gbp3	guanylate binding protein 3	55932	1.6	1.8	1.2	1.6	-	-
Gbp4	guanylate binding protein 4	17472	2.1	2.7	1.3	2.4	-	-
Gbp5	guanylate binding protein 5	229898	2	2.8	1.3	2.2	1.1	-
Gbp7	guanylate binding protein 7	229900	1.6	2.2	1.2	1.7	-	-
Gbp10	guanylate-binding protein 10	626578	2.1	3.2	1.3	1.9	-	-
Gbp8	guanylate-binding protein 8	76074	1.3	1.4	1.4	2.1	-	-
Gbp9	guanylate-binding protein 9	236573	1.2	1.9	1	1.3	-	-
Hp	haptoglobin	15439	1.2	1.8	1.7	1.9	1.3	1.3
Hspa1b	heat shock protein 1B	15511	2.3	2.9	2.4	2.5	-	-
H2-Q4	histocompatibility 2, Q region locus 4	15015	1	1.2	-	1.2	-	-
H2-Q6	histocompatibility 2, Q region locus 6	110557	1	1	-	1.1	-	-
H2-T22	histocompatibility 2, T region locus 22	15039	1	1.3	-	-	-	-
H2-T23	histocompatibility 2, T region locus 23	15040	1	1.3	-	1	-	-
Irgm1	immunity-related GTPase family M member 1	15944	1.4	2.3	1.1	1.4	-	-
Irgm2	immunity-related GTPase family M member 2	54396	1	1.7	-	1.2	-	-
Irg1	immunoresponsive gene 1	16365	2.6	2.5	1.9	2.5	1.6	-
Ifi202b	interferon activated gene 202B	26388	2.4	2.6	2.1	2.2	-	-
Ifi204	interferon activated gene 204	15951	3.3	4	3.2	3.9	-	-
Igtp	interferon gamma induced GTPase	16145	1.4	2.2	1	1.8	-	-
Ifitm6	interferon induced transmembrane protein 6	213002	1.8	1.9	2.4	2.3	1.6	1.5
Ifih1 (MDA5)	interferon induced with helicase C domain 1	71586	1.3	2.1	1.2	1.2	-	-
Iigp1	interferon inducible GTPase 1	60440	1.7	2.9	1.2	1.9	-	-
Irf1	interferon regulatory factor 1	16362	-	1.3	-	1.1	-	-
Irf7	interferon regulatory factor 7	54123	1.7	2.9	1.7	1.1	-	-
Ifi35	interferon-induced protein 35	70110	1.1	1.5	1.1	1.2	-	-
Ifi44	interferon-induced protein 44	99899	2	2.5	1.8	1.6	-	-
Ifi44l	interferon-induced protein 44 like	15061	2.1	2.9	2	2	-	-
Ifit1	interferon-induced protein with tetratricopeptide repeats 1	15957	2.2	3.3	2	1.7	-	-
Ifit2	interferon-induced protein with tetratricopeptide repeats 2	15958	1.8	2.5	1.8	1.7	-	-
Il1a	interleukin 1 alpha	16175	1.9	2	2.2	2.3	1.8	-
Il1f9	interleukin 1 family, member 9	215257	1.3	1.6	1.7	1.8	1.2	1
Il1rn	interleukin 1 receptor antagonist	16181	1.3	1.1	-	1.2	-	-
Il12rb1	interleukin 12 receptor, beta 1	16161	2	2.4	1.7	2.1	1.1	-
Il12rb2	interleukin 12 receptor, beta 2	16162	1.2	1.3	1.3	1.3	-	-
Il15	interleukin 15	16168	1.1	1.3	1.2	1	-	-
Il15ra	interleukin 15 receptor, alpha chain	16169	1.8	1.9	1.5	1.8	1.2	-
Il18bp	interleukin 18 binding protein	16068	1.2	1.7	1.3	1.5	-	-
Il2ra	interleukin 2 receptor, alpha chain	16184	1.1	1.2	-	1.3	1.1	-
Il33	interleukin 33	77125	-	1	-	1.1	-	-
Isg15	ISG15 ubiquitin-like modifier	100038882	1.6	2.2	1.5	1.4	-	-
Klrk1	killer cell lectin-like receptor subfamily K, member 1	27007	1.5	1.7	1.6	1.5	-	-
Lgals9	lectin, galactose binding, soluble 9	16859	1.2	1.6	1.1	1.2	-	-
Lgals3bp	lectin, galactoside-binding, soluble, 3 binding protein	19039	1.2	1.7	1.1	-	-	-
Lilrb4	leukocyte immunoglobulin-like receptor, subfamily B, member 4	14728	1.6	1.5	1.7	1.7	1.1	-
Lcn2	lipocalin 2	16819	1.4	1.6	2.2	2.2	1.4	1.7
Ly6a	lymphocyte antigen 6 complex, locus A	110454	1.7	1.9	1.2	1.3	-	-
Ly6c1	lymphocyte antigen 6 complex, locus C1	17067	1.8	2.2	1.9	1.9	-	-
Ly6c2	lymphocyte antigen 6 complex, locus C2	100041546	1.3	1.6	1.3	1.1	-	-
Ly6g	lymphocyte antigen 6 complex, locus G	546644	-	1.7	2.5	2.2	-	-
Msr1	macrophage scavenger receptor 1	20288	2.3	2	2	2.2	1.4	1.1
Mmp13	matrix metallopeptidase 13	17386	2.7	2.6	2.4	2.4	1.5	-
Mmp19	matrix metallopeptidase 19	58223	1.9	2	2	2	1.5	1.2
Mmp25	matrix metallopeptidase 25	240047	-	1	-	1.1	-	-
Mmp8	matrix metallopeptidase 8	17394	2.7	3	3.1	3.4	2.5	2.8
Ms4a4a	membrane-spanning 4-domains, subfamily A, member 4A	666907	2.6	2.7	2.6	2.5	1.5	1.1
Ms4a4c	membrane-spanning 4-domains, subfamily A, member 4C	64380	1.2	1.5	1.2	1	-	-
Ms4a4d	membrane-spanning 4-domains, subfamily A, member 4D	66607	1.6	1.5	1.6	1.8	-	-
Ms4a6c	membrane-spanning 4-domains, subfamily A, member 6C	73656	1.1	1.1	1.2	1.2	-	-
Ms4a6d	membrane-spanning 4-domains, subfamily A, member 6D	68774	3.2	3.5	3.1	3.3	-	-
Ms4a7	membrane-spanning 4-domains, subfamily A, member 7	109225	1.7	1.4	2	1.9	1.4	-
Mlkl	mixed lineage kinase domain-like	74568	2.1	2.7	2	2.3	-	-
Mnda	myeloid cell nuclear differentiation antigen	381308	1.8	1.8	1.6	1.7	-	-
Mndal	myeloid nuclear differentiation antigen like	1E+08	1.1	1.3	1.1	1.2	-	-

Mx1	myxovirus (influenza virus) resistance 1	17857	3.2	3.9	3	2.8	-	-
Mx2	myxovirus (influenza virus) resistance 2	17858	2.7	3.7	2.1	2.2	-	-
Nampt	nicotinamide phosphoribosyltransferase	59027	1.7	1.9	1.5	2	-	-
Nmi	N-myc (and STAT) interactor	64685	1.3	1.6	1.1	1.5	-	-
Prf1	perforin 1 (pore forming protein)	18646	1.2	1.4	1.1	1.2	-	-
Phf11a	PHD finger protein 11A	219131	1.2	1.4	1.1	1.1	-	-
Phf11b	PHD finger protein 11B	236451	2	1.8	1.7	1.8	-	-
Phf11c	PHD finger protein 11C	628705	2	2.4	1.7	1.7	-	-
Phf11d	PHD finger protein 11D	219132	2.4	2.9	2.4	2.4	1.1	-
Plac8	placenta-specific 8	231507	1.3	1.7	-	1.3	-	-
Parp10	poly (ADP-ribose) polymerase family, member 10	671535	1.2	1.5	-	1.2	-	-
Parp12	poly (ADP-ribose) polymerase family, member 12	243771	1.5	2.5	1.3	1.6	-	-
Parp14	poly (ADP-ribose) polymerase family, member 14	547253	1.1	1.7	-	1	-	-
Parp9	poly (ADP-ribose) polymerase family, member 9	80285	1.3	1.9	1.1	1.2	-	-
Psme1	proteasome (prosome, macropain) 28 subunit, alpha	19186	-	1.1	-	1	-	-
Psma7	proteasome (prosome, macropain) subunit, alpha type 7	26444	-	1.1	-	1.1	-	-
Psmb10	proteasome (prosome, macropain) subunit, beta type 10	19171	1.1	1.4	-	1.3	-	-
Psmb8	proteasome (prosome, macropain) subunit, beta type 8 (large multifunctional peptidase 7)	16913	-	1.4	-	1.1	-	-
Pyhin1	pyrin and HIN domain family, member 1	236312	1.7	1.8	1.5	1.4	-	-
Pydc3	pyrin domain containing 3	100033459	1.9	2.4	1.7	1.5	-	-
Pydc4	pyrin domain containing 4	623121	2.9	3.4	2.2	1.9	-	-
Ppa1	pyrophosphatase (inorganic) 1	67895	1.3	2.1	-	1.5	-	-
Pdk4	pyruvate dehydrogenase kinase, isoenzyme 4	27273	1.5	1.1	1.7	1.4	1.8	1.8
Rtp4	receptor transporter protein 4	67775	1.4	2.2	1.2	1.1	-	-
Retnlg	resistin like gamma	245195	1.1	1.3	1.5	1.6	1.4	1.6
Rnf19b	ring finger protein 19B	75234	-	1.2	-	1.1	-	-
Rnf213	ring finger protein 213	672511	1.4	2.2	1	1	-	-
Slfn1	schlafen 1	20555	1.8	1.9	1.3	1.5	-	-
Slfn3	schlafen 3	20557	1.3	1.7	1.5	1.7	-	-
Slfn4	schlafen 4	20558	1.9	3	2	1.8	-	-
Slfn5	schlafen 5	327978	1.5	2.4	1.4	1.1	-	-
Slfn9	schlafen 9	237886	1.5	2.4	1.4	1.7	-	-
Serpina3f	serine (or cysteine) peptidase inhibitor, clade A, member 3F	238393	1.9	2.7	1.3	2.2	1.3	-
Serpinb9	serine (or cysteine) peptidase inhibitor, clade B, member 9	20723	1.3	1.1	1.1	1.4	-	-
Serpinb9b	serine (or cysteine) peptidase inhibitor, clade B, member 9b	20706	1.2	1	1.1	-	-	-
Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1	18787	1.1	-	-	1	-	-
Stat2	signal transducer and activator of transcription 2	20847	1.4	1.9	1.2	1.5	-	-
Slamf8	SLAM family member 8	74748	-	1.1	-	1.2	-	-
Slc15a3	solute carrier family 15, member 3	65221	-	1.3	-	1.1	-	-
Slc25a22	solute carrier family 25 (mitochondrial carrier, glutamate), member 22	68267	-	1.2	1	1.2	-	-
Socs1	suppressor of cytokine signaling 1	12703	1.6	2.2	-	1.9	-	-
Socs2	suppressor of cytokine signaling 2	216233	1	1.4	-	1.8	-	-
Tgtp1	T cell specific GTPase 1	21822	-	1.4	-	1.1	-	-
Tgtp2	T cell specific GTPase 2	1.00E+08	1.6	2.7	-	1.5	-	-
Trex1	three prime repair exonuclease 1	22040	-	1.2	-	1	-	-
Timp1	tissue inhibitor of metalloproteinase 1	21857	2.8	2.9	2.4	2.9	1.8	1.6
Tlr13	toll-like receptor 13	279572	1.4	1	1.7	1.5	1	-
Tlr3	toll-like receptor 3	142980	1	1.4	1.2	1.1	-	-
Tlr7	toll-like receptor 7	170743	1.1	1.2	1.2	-	-	-
Tlr8	toll-like receptor 8	170744	1.1	1.1	1.3	1	-	-
Trafd1	TRAF type zinc finger domain containing 1	231712	1.1	1.6	1	1.1	-	-
Trem3	triggering receptor expressed on myeloid cells 3	58218	1.1	1.4	1.2	1.5	-	-
Trim12c	tripartite motif-containing 12C	319236	-	1.5	1.2	1.3	-	-
Trim21	tripartite motif-containing 21	20821	1.1	1.3	-	1.4	-	-
Trim30a	tripartite motif-containing 30A	20128	1.3	2.1	-	1.1	-	-
Trim30c	tripartite motif-containing 30C	434219	2.6	3.4	2.5	2.2	-	-
Trim30d	tripartite motif-containing 30D	209387	3.1	3.5	3.4	2.7	1.4	-
Wars	tryptophanyl-tRNA synthetase	22375	1.1	1.6	-	1.4	-	-
Tnfsf10	tumor necrosis factor (ligand) superfamily, member 10	22035	2	2.3	2	1.9	-	-
Usp18	ubiquitin specific peptidase 18	24110	2.2	3.1	2	1.8	-	-
Zbp1	Z-DNA binding protein 1	58203	1.9	2.7	1.3	1.6	-	-

Italics : Genes induced by LSDV alone.
Italics and underlined: Genes induced by CNPV, FWPV and MVA only, and not LSDV.
Bold: Genes induced by Avipoxviruses only (CNPV, FWPV, FeP2 and/ or PEPV)
Bold and Italics: Genes induced by FWPV only.
Bold and underlined: Genes induced by CNPV only.
Underlined: Genes induced by LSDV and MVA, but not by the Avipoxviruses

Selected down regulated genes involved in the immune response are listed in Table 5.3. Full lists of down regulated genes in response to each virus are given in appendix 4. MVA and LSDV induced the down regulation of several genes that were not affected in APV-infected spleens. These included three forms of the chemokine CCL21 (Ccl21a, Ccl21b, Ccl21c) which are potent chemoattractants for lymphocytes and dendritic cells [369] (Table 5.3). Furthermore, MVA and LSDV down regulate the high affinity IgM and IgA Fc (Fragment, crystallizable) receptor Fcamr. Fcamr is the receptor for the Fc fragment of immunoglobulins IgA and IgM [370]. Interestingly, MVA, LSDV, FWPV and CNPV all down regulate the gene encoding the murine homolog for DC-specific ICAM-3–grabbing nonintegrin (DC SIGN) (Cd209a), and MVA and LSDV down regulate an additional DC SIGN homolog, CD209b (SIGNR1) (Table 5.3). LSDV uniquely down regulates CD59a, which is the primary regulator of complement membrane attack in mouse [371] and CD7 which is expressed on T- and NK cells, and on cells in the early stages of T, B, and myeloid cell differentiation [372]. LSDV also uniquely down regulates the immunoglobulin kappa chain complex (IgK) amongst other immune related genes (Table 5.3). TLR11 is down regulated by MVA alone (Table 5.3).

While many of the immunity related genes listed in Table 5.2 are regulated in some way by Type I IFNs, in order to characterise and compare the differences in the Type I IFN-regulated responses between MVA, LSDV, FWPV, CNPV, FeP2 and PEPV at 24hrs, we analysed a selection of genes known to be involved in the IFN response [373-375] (Fig. 5.4). This figure clearly shows that LSDV induces the greatest IFN response compared to the other viruses (Fig. 5.4).

MVA, FWPV and CNPV all up regulate the protease caspase 1 whereas LSDV does not (Fig. 5.4). MVA, FWPV, CNPV and LSDV significantly up regulated caspase 4 (casp 4) (historically called caspase 11 in the mouse) (Table 5.2). The SA APVs, FeP2 and PEPV do not regulate any caspase genes.

The up- and down regulated genes involved in B cell and T cell responses induced by the viruses in this study were compared (Fig. 5.5). Fig. 5.5A highlights the regulated genes that are involved in the T cell response. FeP2 and PEPV regulated only one gene each involved in this response, Ctla2a and MHC class I gene, H2-M2, respectively. The other four viruses differentially regulated several MHC class I genes amongst others (Fig. 5.5A). As highlighted in Table 2, the APVs CNPV and FWPV exclusively up regulate immunoglobulin heavy chain genes, (Ighg (IgG) and

Ighg3 (IgG3)) with CNPV inducing a third, Ighm (IgM) (Fig.5B). LSDV down regulates the immunoglobulin kappa (IgK) chain complex (Fig. 5.5B). In addition to these, the poxviruses differentially regulate genes for Fc receptors and complement genes (Fig. 5.5B).

Here we compared the gene expression induced by each of the 6 poxviruses at 24hrs to selected correlates of protection and molecular signatures from previously published studies (Table 5.4). The induction of multiple PRRs has been shown to activate different immune pathways and thereby induce a more polyvalent immune response [376, 377]. We identified differential expression of several genes involved in pathogen recognition (Table 5.4). Several genes are common to the innate and adaptive immune responses induced by the poxviruses analysed here and other viral vectors analysed elsewhere (Table 5.4).

5.4 Discussion

In this study, we compared the gene expression profiles in mouse spleens after infection with poxviruses from 3 different genera. Although *in vitro* expression studies have provided useful information, gene expression profiles performed in cell culture may not accurately reflect the changes that occur as result of infection *in vivo.* We have therefore made use of a mouse model to delineate the host responses to host-restricted poxviruses in a whole organism. Here we show that six host-restricted poxviruses produce different gene expression profiles (Fig. 5.1), including GO term enrichment (Fig. 5.3) and genes responsible for immune responses (Table 5.2). These distinct profiles indicate potentially significant biological differences between these viruses.

Table 5.3. Selection of down regulated genes in mouse spleens in response to MVA, LSDV, CNPV, FWPV, FeP2 and PEPV. Differences in Log_2 Fold Changes (between each virus and the control) are depicted.

Symbol	Name	Entrez	MVA	LSDV	CNPV	FWPV	PEPV	FeP2
GENES DOWN REGULATED BY LSDV ALONE								
Adamdec1	*ADAM-like, decysin 1*	*58860*	-	*-1.1*	-	-	-	-
Cd59a	*CD59a antigen*	*12509*	-	*-1.2*	-	-	-	-
Cd7	*CD7 antigen*	*12516*	-	*-1.1*	-	-	-	-
Esm1	*endothelial cell-specific molecule 1*	*71690*	-	*-1.1*	-	-	-	-
Igfbp3	*insulin-like growth factor binding protein 3*	*16009*	-	*-1*	-	-	-	-
Igk	*immunoglobulin kappa chain complex*	*243469*	-	*-1*	-	-	-	-
Lilra5	*leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 5*	*232801*	-	*-1.1*	-	-	-	-
Prkcg	*protein kinase C, gamma*	*18752*	-	*-1*	-	-	-	-
GENES DOWN REGULATED BY MVA ALONE								
Ctsf	**cathepsin F**	**56464**	**-1.1**	**-**	**-**	**-**	**-**	**-**
Depdc1a	**DEP domain containing 1a**	**76131**	**-1.1**	**-**	**-**	**-**	**-**	**-**
Diap3	**diaphanous homolog 3 (Drosophila)**	**56419**	**-1.1**	**-**	**-**	**-**	**-**	**-**
Hmmr (CD168)	**hyaluronan mediated motility receptor (RHAMM)**	**15366**	**-1**	**-**	**-**	**-**	**-**	**-**
Tlr11	**toll-like receptor 11**	**239081**	**-1**	**-**	**-**	**-**	**-**	**-**
GENES INDUCED BY LSDV AND MVA, BUT NOT BY CNPV AND FWPV								
Ccl21a	chemokine (C-C motif) ligand 21A (serine)	18829	-1.3	-1.7	-	-	-	-
Ccl21b	chemokine (C-C motif) ligand 21B (leucine)	100042493	-1.2	-1.7	-	-	-	-
Ccl21c	chemokine (C-C motif) ligand 21C (leucine)	65956	-1.2	-1.6	-	-	-	-
Kel	Kell blood group	23925	-1.5	-1.5	-	-	-	-
Slc12a2	solute carrier family 12, member 2	20496	-1	-1	-	-	-	-
Timd4	T cell immunoglobulin and mucin domain containing 4	276891	-1.1	-1.3	-	-	-	-
GENES INDUCED BY CNPV, FWPV AND MVA ONLY, AND NOT LSDV.								
Tspan33	*tetraspanin 33*	*232670*	*-1.6*	-	*-1.3*	*-1.1*	-	-
OTHER								
Abca9	ATP-binding cassette, sub-family A (ABC1), member 9	217262	-1.3	-1.5	-	-1.1	-	-
Aplnr	apelin receptor	23796	-1.4	-1.5	-1.2	-1.2	-	-
Cd209a	CD209a antigen	170786	-1.7	-2.2	-1.1	-1.5	-	-
Cd209b	CD209b antigen	69165	-1.2	-1.3	-	-	-	-
Cldn13	claudin 13	57255	-1.5	-1.2	-1.1	-	-	-
Emr4	EGF-like module containing, mucin-like, hormone receptor-like sequence 4	52614	-1.4	-1.8	-1.1	-1.5	-1.3	-
Fcamr	Fc receptor, IgA, IgM, high affinity	64435	-1.1	-	-1.5	-	-	-
Fcer2a	Fc receptor, IgE, low affinity II, alpha polypeptide	14128	-2.6	-3	-2.4	-2.5	-2.2	-1.3
H2-M2	histocompatibility 2, M region locus 2	14990	-1	-1.4	-1	-1	-1.1	-
Hs3st2	heparan sulfate (glucosamine) 3-O-sulfotransferase 2	195646	-1.4	-1.3	-1	-1	-	-
Ifi27l1	interferon, alpha-inducible protein 27 like 1	52668	-1.4	-1.3	-	-	-	-
Mgst3	microsomal glutathione S-transferase 3	66447	-1.6	-1	-	-1	-	-
Slc16a10	solute carrier family 16 (monocarboxylic acid transporters), member 10	72472	-1.4	-	-	-1.1	-	-
Slc2a4	solute carrier family 2 (facilitated glucose transporter), member 4	20528	-1.7	-1	-	-1.1	-	-
Slc38a5	solute carrier family 38, member 5	209837	-1.4	-1.2	-1	-	-	-
Slc6a20a	solute carrier family 6 (neurotransmitter transporter), member 20A	102680	-1.4	-1.3	-1	-1	-	-
Tfrc	transferrin receptor	22042	-1.2	-	-	-	-	-
Tspan8	tetraspanin 8	216350	-1.4	-	-	-1	-	-

Italics **: Genes induced by LSDV alone.**

Bold**: Genes induced by MVA alone**

Underlined: Genes induced by LSDV and MVA, but not by avipoxviruses

Italics and underlined**: Genes induced by CNPV, FWPV and MVA only, and not LSDV.**

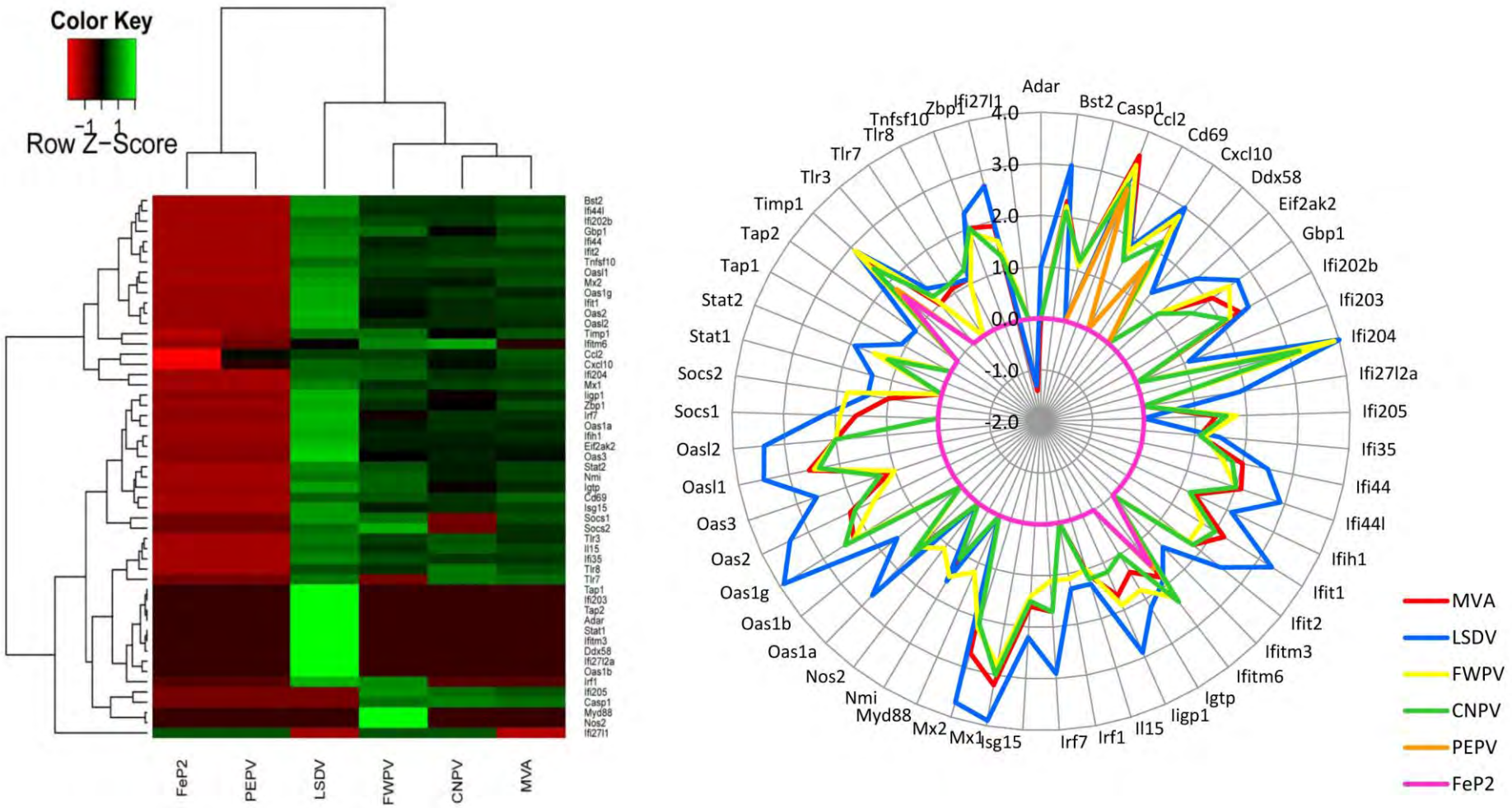


Figure 5.4. Heatmap (A) and radial plot (B) depicting the differences in the type I Interferon response induced by CNPV, FeP2, FWPV, LSDV, MVA and PEPV. Figure 4A represents the $\log_2$ fold change (FC) induction of the different genes up regulated in the four samples compared to the control. A $\log_2$FC of 0 is given where genes are not present over the cut off (±1). Unsupervised hierarchical clustering of the samples is represented by dendograms. Clustering analysis and heatmap was performed in the R package, gplots (Warnes, 2009). **Figure 4B** shows a radial plot depicting the magnitude and breadth of the type I interferon response induced by the four viruses. The distance from the centre of the plot indicates log2-fold change (ranging from -2 to 4).

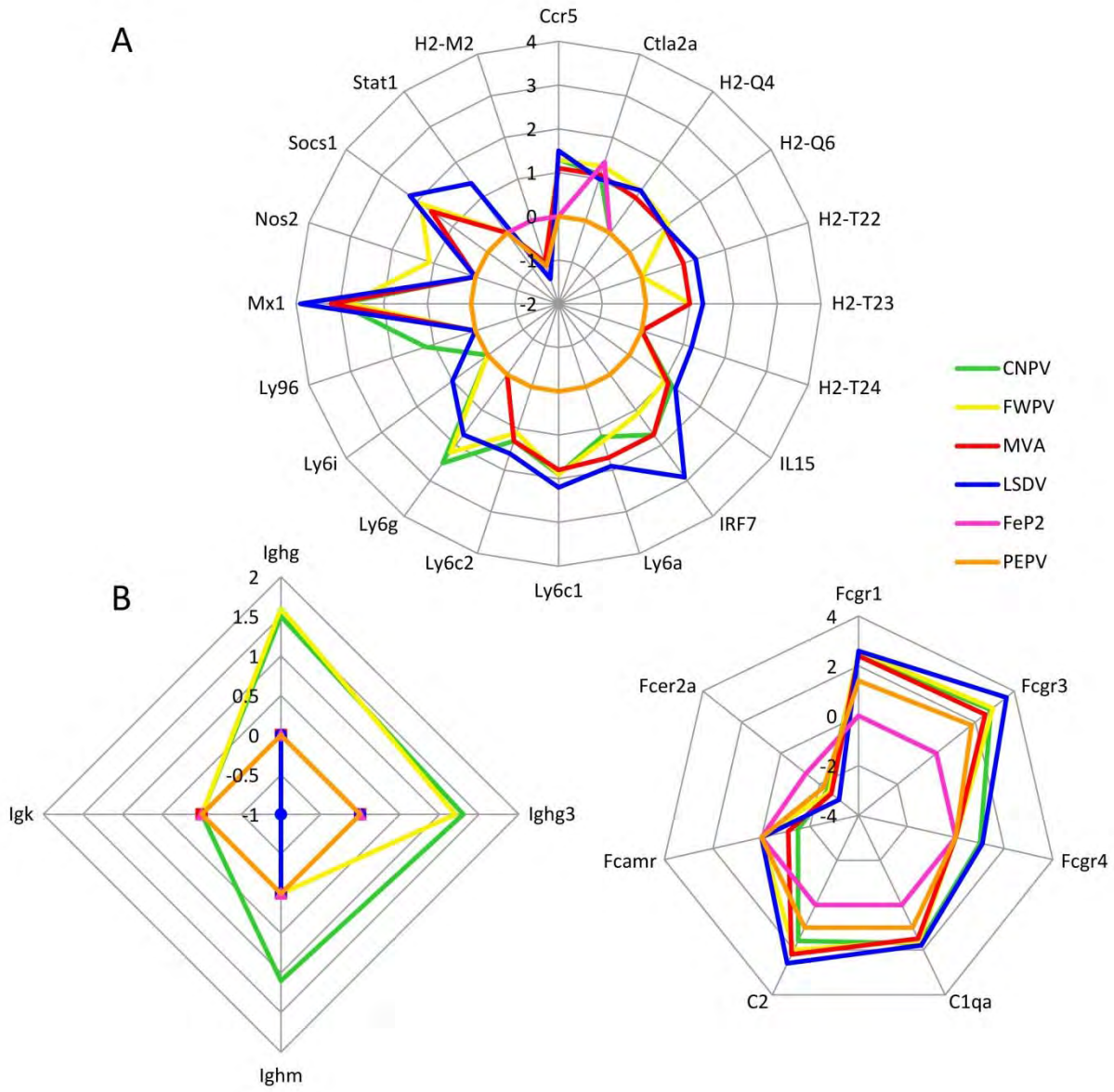


Figure 5.5A). T-cell specific responses and 5B) B-cell specific responses up- or down regulated in mouse spleens in response to CNPV, FeP2, FWPV, LSDV, MVA and PEPV. The $\log_2$ fold changes of significantly differentially expressed (p value ≤ 0.05) genes involved in the respective types of responses are compared. A value of 0 indicates that no change was observed compared to mock-infected mouse spleens. A positive value depicts up regulated genes and a negative value depicts down regulated genes.

Table 5.4. Comparison of early poxvirus-induced immune responses to selected innate molecular signatures of existing vaccine vectors. Differences in Log_2 Fold Changes (between each virus and the control) are depicted.

	description	MVA	LSDV	CNPV	FWPV	PEPV	FeP2	Evidence	References
INNATE IMMUNE RESPONSE									
Pathogen recognition									
Tlr13	toll-like receptor 13	1.4	1.0	1.7	1.5	1.0	-		
Tlr3	toll-like receptor 3	1	1.4	1.2	1.1	-	-	Merck Ad5/HIV	[344]
Tlr7	toll-like receptor 7	1.1	1.2	1.2	-	-	-	YF-17D, LAIV	[342, 343]
Tlr8	toll-like receptor 8	1.1	1.1	1.3	1.0	-	-	Merck Ad5/HIV	[344]
Tlr11	toll-like receptor 11	-1.0	-	-	-	-	-		
Ddx58 (RIG-I)	RIG-I-like receptor	-	1.3	-	-	-	-	YF-17D	[342]
Cd209a (DC SIGN)	CD209a antigen	-1.7	-2.2	-1.1	-1.5	-	-		
Cd209b (DC SIGN)	CD209b antigen	-1.2	-1.3	-	-	-	-		
Ifih1 (MDA5)	RIG-I-like receptor	1.3	2.1	1.2	1.2	-	-	YF-17D	[342]
Zbp1 (DAI)	cytoplasmic double-stranded DNA sensor	1.9	2.7	1.3	1.6	-	-		
Dhx58 (LGP2)	RIG-I-like receptor	1.7	2.5	1.6	1.5	-	-	YF-17D	[342]
Eif2ak2 (PKR)	eukaryotic translation initiation factor 2-alpha kinase 2 (protein kinase R)	1.1	2.1	1.1	1.1	-	-	YF-17D	[342]
Genes associated with the innate immune response of viral vectors									
Cxcl10 (IP-10)	chemokine (C-X-C motif) ligand 10	2.7	3	2.2	2.8	1.7	-	Significantly upregulated in response to YF-17D, Merck Ad5/HIV, TIV	[344]
Mx1	myxovirus (influenza virus) resistance 1	3.2	3.9	3	2.8	-	-	YF-17D	[342]
Il-1α	interleukin 1 alpha	1.9	2	2.2	2.3	1.8	-	Significantly upregulated in response to YF-17D	
Isg15	ISG15 ubiquitin-like modifier	1.6	2.2	1.5	1.4	-	-	Merck Ad5/HIV	[344]
Stat1	signal transducer and activator of transcription 1	-	1.4	-	-	-	-	YF-17D, Merck Ad5/HIV, LAIV	[342-344]
Cxcl11 (I-TAC)	chemokine (C-X-C motif) ligand 11	4.5	4.4	3.4	4.3	1.5	-	Merck Ad5/HIV	[344]
Ccr5	chemokine (C-C motif) receptor 5	1.1	1.5	1.3	1.3	-	-	Merck Ad5/HIV	[344]
Gbp7	guanylate binding protein 7	1.6	2.2	1.2	1.7	-	-	Merck Ad5/HIV	[344]
Irf1	interferon regulatory factor 1	-	1.3	-	1.1	-	-	Merck Ad5/HIV	[344]
Stat2	signal transducer and activator of transcription 2	1.4	1.9	1.2	1.5	-	-	LAIV	[343]
Irf7	interferon regulatory factor 7	1.7	2.9	1.7	1.1	-	-	LAIV	[343]
Casp1	caspase 1	1.1	-	1.2	1.1	-	-		
ADAPTIVE IMMUNE RESPONSE									
B cell related responses									
Ighg	Immunoglobulin heavy chain (gamma polypeptide)	-	-	1.5	1.6	-	-		
Ighg3	Immunoglobulin heavy constant gamma 3	-	-	1.3	1.2	-	-	TIV, correlated with decreased risk of HIV-1 infection in the RV144 trial ALVAC-HIV(vCP1521)	[378]
Ighm	immunoglobulin heavy constant mu	-	-	1.1	-	-	-	positively correllates with antibody response to TIV	[343]
Igk	immunoglobulin kappa chain complex	-	-1.0	-	-	-	-	positively correllates with antibody response to TIV	[343]
T cell related responses									
Gzmb	granzyme B	3.7	4.7	4.1	4.2	2.4	-	expressed by CD8+ T cells in response to YF-17D	[342]
Ccr5	chemokine (C-C motif) receptor 5	1.1	1.5	1.3	1.3	-	-	expressed by CD8+ T cells in response to YF-17D	[342]
Ccl2 (MCP1)	chemokine (C-C motif) ligand 2	3.5	3.3	2.9	3.3	2.8	-	predicted the magnitude of the CD8+ T cell response to Merck Ad5/HIV	[344]

Abbreviations: Human immunodeficiency virus (HIV), Live attenuated influenza vaccine (LAIV), Trivalent influenza vaccine (TIV), Yellow fever vaccine (YF-17D), Merck's Adenovirus subtype 5-based HIV vaccine (Merck Ad5/HIV)

Previous *in vitro* studies with VACV, MVA and NYVAC have resulted in more down regulated genes than up regulated genes [87-90]; however, this was not the case in other poxviruses, including CPV [92], MXPV [92] or ALVAC [91]. Unlike our study done in a mouse model all these studies were done in cell culture. We show that fewer genes are down regulated than up regulated in response to *in vivo* infection at 24hrs (Table 5.1) and that distinct differences exist between the genes that are down regulated in response to the six viruses. A list of selected down regulated genes known to be involved in the immune response is given in Table 5.3. All genes that are down regulated in response to infection of mice are listed in appendix 4. For each poxvirus tested several mouse genes dysregulated are not yet annotated suggesting biological roles for unannotated genes and highlighting the importance of further functional analysis and annotation of the mouse genome.

Contrary to *in vitro* studies, MVA caused more transcripts to be up regulated than down regulated in mouse spleens. However, MVA infection resulted in a greater number of down regulated transcripts compared to LSDV, FWPV and CNPV. MVA caused the down regulation of several genes involved in pyruvate metabolism and cell division. VACV is known to inhibit the synthesis and reduce the stability of host RNA [379, 380] and the down regulation of cellular genes by MVA, both in mice and *in vitro* [87] may represent a similar process. Interestingly, MVA specifically down regulates murine-only TLR11, which recognises uropathogenic bacteria [381] and a profilin-like molecule from the protozoan parasite Toxoplasma gondii [382]. TLR11 has been shown to be reduced in response to conditions of stress [383].

MVA and LSDV induced the down regulation of several genes that were not affected by the APVs tested. These included three forms of the chemokine CCL21 (Ccl21a, Ccl21b, Ccl21c) which are potent chemoattractants for lymphocytes and dendritic cells [369] (Table 5.3). VACV A41L encodes a chemokine binding protein which binds and inhibits CCL21 [384]. VACV and MVA deletion mutants lacking the A41L gene, induce stronger virus-specific CD8+ T-cell responses [384, 385]. LSDV does not have a homolog of the A41L gene; there must be other mechanisms that mammalian poxviruses have evolved to evade the effects of CCL21, which is clearly important for the host in clearing poxvirus infection. In selecting/designing a vaccine vector it would be desirable to use a virus which lacks A41L and does not down regulate CCL21 if a strong CD8+ T cell response is required. Interestingly, MVA, LSDV, FWPV and CNPV all down regulated the gene encoding the murine homolog for DC-specific ICAM-3–grabbing nonintegrin (DC SIGN) (Cd209a). Furthermore

MVA, LSDV and FWPV down regulated an additional DC SIGN homolog, CD209b (SIGNR1) (Table 5.3). DC-SIGN is a C-type lectin surface receptor found on dendritic cells (DCs) that interacts with intracellular adhesion molecule-3 (ICAM-3) to facilitate the clustering of DCs and T-cells necessary for T-cell activation [386]. Down regulation of DC-SIGN has been observed in response to infection with human herpesvirus 6 [386].

All six viruses down regulated the Fcer2a gene which encodes the IgE FC receptor alpha polypeptide. Fc receptors bind to antibodies on infected cells stimulating antibody-mediated phagocytosis or antibody-dependent cell-mediated cytotoxicity (ADCC). The Fc epsilon receptor II, unlike the other FC receptors is a c-type lectin and exists in two forms, with the alpha form (Fcer2a) functioning in B cells as an adhesion molecule [387, 388]. Most poxviruses encode several c-type lectin-like molecules [24, 25, 67] which are important, either in the host control of infection (and the virally encoded c-type lectin proteins act as decoys) or for virus processes. The LSDV genome lacks a recognisable c-type lectin homolog [187]. It is not clear how poxviruses down regulate Fcer2a and whether this is an advantage or disadvantage for a vaccine vector. On the one hand the vector would not be cleared in an ADCC manner; however, down regulation of Fcer2a may also limit the desired (ADCC) response to a foreign gene product.

LSDV caused the most significant response in mice compared to the other poxviruses, both in terms of the number of up regulated genes and the magnitude and breadth of the type I IFN response (Fig. 5.5). According to unsupervised hierarchical clustering (Fig. 5.1), LSDV clustered independently from the APVs and MVA. LSDV up regulated genes involved in the antigen processing and presentation pathway (H2-T24, Tap1 and Tap2). Furthermore, LSDV uniquely up regulated the gene encoding macrophage migration inhibitory factor (Mif), which is important in both macrophage function and T-cell immunity [389], and Ddx58, otherwise known as RIG-I (retinoic acid-inducible gene 1), which recognises viral RNA, activating downstream signalling pathways that facilitate type I IFN production [390]. The up regulation of RIG-I may, in part, be responsible for the increased type I IFN response seen in LSDV-infected mice. Another one of the many genes uniquely up regulated by LSDV was the transcription factor (Myc) that promotes growth, proliferation and apoptosis [391]. Myc has been shown to be up regulated in response to infection with NYVAC and MVA in HeLa cells [89]. The absence of Myc up regulation in mouse spleens by MVA was surprising.

Both LSDV and MVA up regulated a cellular homolog of Moloney Leukemia Virus 10 (Mov10), which has been shown to inhibit retrovirus replication [392]. Mov10 overexpression can reduce the infectivity of human immunodeficiency virus type 1 (HIV-1), simian immunodeficiency virus, and murine leukemia virus [392]. It specifically interacts with the nucleocapsid domain of HIV Gag [392], which may have implications for vaccine vectors encoding Gag proteins. APVs may therefore be better vectors than MVA or LSDV for the expression of Gag.

Amongst the four APVs analysed here, FWPV induced the strongest host response in mice whereas FeP2 infection resulted in remarkably little change in host gene expression. The vast difference in the host responses between APVs is expected between CNPV and FWPV, as on a genomic level, these viruses are significantly divergent with amino acid identity between ORF homologues (55-74%) being similar to that observed between different ChPV genera [24, 24]. It was suprising to see differences between the host responses induced by FeP2 and PEPV which share 94.4% nucleotide identity with each other and 85.3% and 84.0% nucleotide identity with FWPV respectively (The PEPV sequence is to be presented in Olivia Carulei‘s PhD dissertation). Since APVs are restricted to only avian hosts, one would expect to observe fewer differences in mammalian host responses induced by them as it is highly likely that their proteins are not as functional in mammalian cells as those of MVA and LSDV. Here we show that three relatively closely related APVs (FWPV, FeP2 and PEPV) induce significant differences in gene expression in the host. Unsupervised hierarchical clustering differentiates between the observed responses to the four APVs, grouping CNPV and MVA together and FWPV in a separate cluster (more closely related to CNPV and MVA than to LSDV) (Fig. 5.1). FeP2 and PEPV group together in a cluster that is separate from the other four viruses (Fig. 5.1). This grouping is quite different from phylogenetic relationships established by DNA sequence comparisons (chapter 4, Fig. 4.1).

CNPV and FWPV induce the up regulation of two immunoglobulin genes (Ighg and Ighg3 (IgG3)) with CNPV up regulating a third, Ighm (Fig. 5.5B). Antibodies of the same epitope specificity but of a different subclass can have different antibody effector functions [378]. In a recent comparison of the immune responses resulting from the partially effective clinical RV144 HIV-1 trial and the ineffective VAX003 trial, it was shown that HIV-1–specific IgG3 antibodies distinguished the outcomes of the two HIV-1 vaccine trials (RV144 and VAX003) and correlated with decreased risk of HIV-1 infection in the RV144 trial. It is suggested that the CNPV, ALVAC-HIV

(vCP1521) prime component of RV144 may have stimulated different antibody subclasses, specifically IgG3, compared to the protein-only vaccine (VAX003) [378]. The up regulation of IgG3 specifically by FWPV and CNPV *in vivo,* suggests that these two APV vectors may be involved in stimulation of the clinically important IgG3 antibody subclass. Up regulation of IgG3 was not detected in ALVAC-infected monocyte derived dendritic cells (MDDCs) [91]; this potentially significant finding is an example of the importance of *in vivo* testing.

CNPV infection induces the unique up regulation of immunoglobulin heavy constant mu (Ighm). Ighm is the gene encoding the heavy chain of IgM. IgM is the first antibody isotype induced in response to infection or vaccination [393]. IgM antibodies are multi-faceted in their antimicrobial function and are involved in direct neutralization by particle agglutination, complement activation, enhancement of phagocytosis and the generation of the adaptive immune response [394]. There are two classes of IgM, natural (innate) IgM, and immune (adaptive/induced) IgM [393]. Induced IgM is produced primarily by spleen and lymph node B-2 cells, following antigenic exposure [395]. Vaccine induced IgM has been shown to contribute to the early control of Rabies virus [394] and induced, but not natural IgM protects against West Nile virus [395]. IgM has been proposed for use as a vaccine adjuvant [396] and its up regulation by a vector backbone may be beneficial to vaccine efficacy.

The fact that LSDV uniquely down regulates the immunoglobulin (light chain) kappa chain complex (IgK) and the absence of any up regulation of immunoglobulin genes suggests that this virus may be a poor inducer of an antibody response. However, several LSDV-based vaccines have been shown to induce vaccine insert-specific antibody responses [338, 397, 398].

Type I IFN responses have been highlighted in previous studies investigating host gene expression changes in response to different host-restricted poxviruses [88, 91]. Type I IFN induces an extensive range of ISGs with various anti-viral functions (reviewed here: [108]). Poxviruses, in turn, encode several immunoregulatory proteins, which counteract these host anti-viral responses [156]. Upon passage without immune pressure, during the process of attenuation of vaccines, genes associated with immunoregulation and host range become deleted or mutated, as they serve no function to the virus. Previous studies have shown that *in vitro* MVA infection causes increased innate system activation compared to vaccinia virus Western Reserve (VACV-WR) [87, 90]. Attenuated poxviruses, which have lost the

function of several important immunomodulatory proteins, tend to cause increased stimulation of the host innate immune response. This is confirmed by the study in which the adaptive and memory responses induced by the HIV vaccine candidate NYVAC-C were enhanced following removal of the Type I and II IFN decoy receptors encoded by the VACV B8R and/or B19R genes [157]. Similarly, in the MVA-based HIV vaccine candidate MVA-B the deletion of the genes C6L and K7R, coding for inhibitors of IFN signalling, improved the resulting adaptive and memory immune responses [158].

In concurrence with previous studies of poxvirus-induced host responses [91][88], Type I IFN responses were initiated by MVA, LSDV, CNPV and FWPV, with LSDV inducing the strongest response in mice (Fig. 5.4), followed by CNPV and FWPV, with MVA inducing a relatively low IFN response. FeP2 and PEPV induced very little ISG expression. As seen in ALVAC-induced gene expression in human MDDC's [91], numerous IFN responsive genes and downstream signalling molecules were up regulated despite the lack of meaningful IFN-β or IFN-α induction. It is possible that the peak of IFN-β or IFN-α levels was missed at 24 hours post infection in which case the experiment should be repeated at earlier time points. Alternatively, altered IFN-β or IFN-α levels may not have been detected due to the relatively small increase in these proteins compared to the amplified levels of ISGs. The observed enhanced type I IFN-specific and other immune responses elicited by LSDV, FWPV and CNPV compared to MVA may be due to the absence of virus-encoded immunomodulators in these viruses which could still be present in MVA.

Comparison of the two attenuated VACV strains, NYVAC and MVA showed MVA to induce a stronger IFN response than NYVAC, as a result of the loss of more IFN-antagonists during its passage in CEFs [44, 67, 87, 89]. Interestingly, although LSDV is host-restricted to cells of mammalian origin (bovine) and FWPV and CNPV to avian cells, LSDV induces a stronger IFN response in mice. Either LSDV host-range proteins are unable to subvert host responses in non-permissive hosts, or, genes encoding such proteins have been deleted/mutated during attenuation of the vaccine. Our results suggest that LSDV is more immunogenic than FWPV and CNPV in mice. It is not known whether this greater IFN-response induced by LSDV in comparison to APVs would lead to enhanced clearance of the virus and a decreased immune response to transgenes, or whether the increased IFN response would result in an improved immune response to the transgene, should LSDV be used as a vaccine vector.

TLRs are important regulators of the innate immune system. Poxviruses are recognized by a number of different PRRs with innate immune sensing patterns differing considerably between species and even between different derivatives of the same parent species (VACV, MVA and NYVAC) [96]. Several studies have implicated different TLRs required for innate recognition of poxviruses. Recombinant FWPV is recognised in a TLR7 and TLR9-dependent manner in DCs; binding to both TLRs results in type I IFN production. [101]. Innate immune recognition of VACV is mediated by TLR2 [99] and TLR2 and TLR6 are important for MVA recognition and subsequent cytokine production [95]. Conversely, TLR3 activation has been shown to contribute to VACV pathogenesis [102] demonstrating that activation of TLRs in poxvirus infection may have a dual role, acting to initiate the host response to infection and as an important mediator of poxvirus pathogenicity.

In our mouse spleen study we show that TLR13, TLR3 and TLR8 are up regulated by four poxviruses analysed (CNPV, FWPV, MVA and LSDV). In addition, TLR7 is up regulated by CNPV, MVA and LSDV but not by FWPV (Table 5.2). TLR13 is a murine intracellular receptor that is predominantly expressed in splenic DC‘s and macrophages and activates both a MyD88- and TAK1-dependent TLR signalling pathway to activate NF-κB, and induces type I IFN through IRF7. TLR13 has been shown to be involved in the recognition of vesicular stomatitis virus [399], but not of poxviruses. TLR7 and 8 both recognise ssRNA [400][401] but have functional differences. TLR7 is more effective at inducing IFN and IFN-regulated molecules, and conversely, TLR8 is more effective at inducing proinflammatory cytokines and chemokines, such as TNF-α, IL-12, and MIP-1 [402]. Up regulation of TLR3, which detects double stranded RNA, has been observed in response to MVA, but not NYVAC infection of MDDCs [88]. Due to the implication of the involvement of the poxvirus gene A52R in TLR signalling and its absence in MVA, this gene was highlighted as potentially responsible for the up regulation of TLR3 [88]. To date, no homolog of this gene has been found in any sequenced APVs [24, 25]; however, several homologs of this gene exist in the LSDV genome [187]. This suggests that either the LSDV genes are not functional in mice or that the absence of an A52R homolog is not solely responsible for the up regulation of TLR3. IFNs have been shown to up regulate TLR gene expression in viral infections [98]. Here we have established that CNPV, FWPV, MVA and LSDV all induce significant type I IFN responses and we suggest that the up regulation of TLR expression may be a result of this.

The important role played by TLRs in both the innate and adaptive immune responses to pathogens is undisputed. TLRs activate signalling pathways to induce appropriate immune system effector responses. Specifically, TLR3, TLR4, TLR7, TLR8 and TLR9 induce antiviral responses by inducing type I IFN responses [97].

MVA, FWPV and CNPV all up regulate the protease caspase 1 whereas LSDV does not (Fig. 5.4). Eukaryotic cells can undergo several distinct programs of cell death, producing different morphological and physiological outcomes. Caspase 1 dependent programmed cell death (pyroptosis), unlike apoptosis, is a pro-inflammatory process that has recently been recognised as important for the control of microbial infections [138]. Caspase 1 is activated in large oligomeric complexes, inflammasomes [403], which assemble in response to the sensing of PAMPs (microbial/viral) [404] or danger or stress signals termed danger-associated molecular patterns (DAMPs) [405, 406]. The up regulated transcription of caspase 1 by MVA, FWPV and CNPV suggests that pyroptosis is taking place in response to infection with these viruses. All of MVA, FWPV, CNPV and LSDV also significantly up regulated caspase 4 (casp 4) (historically called caspase 11 in the mouse). Caspase 4 expression is necessary for the maturation of the proproteins of IL-1b and IL-18 (proIL-1b, proIL-18) and plays an important role in the activation of caspase-1 in inflammasome complexes, and therefore inflammation [407]. The correlation of caspase up regulation with either apoptosis or pyroptosis is still to be assessed.

Application of the systems biology approach to vaccines and determination of innate immune signatures has proven useful in predicting the immunogenicity of the highly effective yellow fever vaccine (YF-17D) [342], the seasonal influenza vaccines [343] and the immunogenic but inefficacious Merck Ad5/HIV vaccine [344]. A better understanding of the mechanisms underlying the optimal innate immune responses would aid rational vaccine development. Here we compared selected key gene signatures involved in the early immune responses of several vaccine vectors and the host gene expression changes induced by the 6 poxviruses. Several of the innate immune signatures observed in tested vaccines, including YF-17D, Merck Ad5/HIV, LAIV (live attenuated influenza vaccine) and TIV (trivalent influenza vaccine), were common to one or more of the poxviruses investigated here. The gene encoding monocyte chemotactic protein 1 (MCP1) (Ccl2) was up regulated by 5 out of the 6 poxviruses (MVA, LSDV, CNPV, FWPV and PEPV). This gene was positively correlated with the CD8+ T cell response to Merck Ad5/HIV

vaccination [344]. Immunoglobulin genes, Ighm (up regulated by CNPV) and IgK (down regulated by LSDV) were positively correlated with the antibody response to TIV influenza vaccination [343]. This suggests that the different poxviruses could be associated with different levels of antibody induction during the adaptive immune response. Based on our data we speculate that LSDV may be more suitable for a T-cell vaccine and CNPV more suitable for the induction of an antibody response. This reflects the published data on LSDV [408] and CNPV [378].

Microarray analyses can provide important information regarding the effect of different clinically relevant viruses on host gene expression. One limitation of microarray data analysis is that as of yet there are no standardised methods of statistical analysis. It has been demonstrated previously that fold change designations and p-value cutoffs can significantly alter microarray interpretation [409]. Here we have chosen stringent fold change and p-value cutoffs (log_2FC ±1, adjusted p-value<0.05) in line with similar studies [87-90], in order to avoid false discovery and inaccurate biological inferences. We concede that in doing so, some smaller changes in gene expression may have been overlooked. Further work should entail investigating gene dysregulation at different times post infection. Also, innate immune signatures should be directly correlated with subsequent adaptive responses. Correlation of gene expression data with biological or clinical findings would be most informative.

The findings presented here indicate that six, genetically diverse host-restricted poxviruses, CNPV, FWPV, FeP2, PEPV, MVA and LSDV, produce qualitatively and quantitatively distinct host responses in an *in vivo* mouse model. This suggests potentially important biological differences in response to infection, especially because small changes in gene expression may produce disproportionate results in biological systems. Since the innate immune system plays a fundamental role in controlling adaptive immune responses [345, 347] these differences may affect the immune response induced to vaccine antigen in vectors based on these viruses.

Chapter 6
Conclusions

In this study, we have tested the hypothesis that there are novel South African APVs that could be developed as vaccine vectors.

Preliminary characterisation of 8 novel South African APV isolates was performed (chapter 2). For the first time, information is available on the APVs that are circulating in South African birds. Although the phylogenetic analysis of highly conserved genetic regions indicates that the SA APVs are closely related to each other, variation in growth characteristics on CAMs suggests that many of our novel APVs differ significantly.

When grown on chick CAMs, FeP2 produced white, variably-sized pocks and caused a substantial amount of membrane thickening (chapter 2, Fig. 2.2) with pronounced immune infiltration and angiogenesis of the mesoderm (chapter 2, Fig 2.3). This growth phenotype was markedly different from that observed for FWPV (vaccine strain DCEP 25), which produced less membrane thickening and red, haemorrhagic pocks, and CNPV (wild type) which caused no obvious membrane thickening and produced small, yellow pocks (chapter 2, Table 2.2 and 2.3, Fig. 2.2). Therefore, according to growth phenotype on CAMs, we concluded that FeP2 was different from both CNPV and FWPV. In addition, according to phylogenetic analysis, FeP2 grouped separately from CNPV and FWPV (in subclade A3c). Moreover, FeP2 grouped differently according to which conserved genetic region was analysed (chapter 2, Fig 2.4-2.7). This suggested that there would be significant differences in the less conserved regions of the FeP2 genome. Preliminary attempts to grow the novel SA APVs in primary CEF cells suggested that FeP2 and not the other virises would grow in cell culture. From these results we chose FeP2 for further characterisation.

The full genome sequence of FeP2 was determined (chapter 4) and shown to be significantly different from that of CNPV (approx. 63% identity) and FWPV (approx. 85% identity). Based on DNA similarity of the FeP2 and FWPV genomes, as well as the differences in phylogenetics and growth characteristics between these two viruses (chapters 2 and 3), we conclude that FeP2 is a separate species of APV.

The most striking difference between FeP2 and FWPV is a large deletion of ~16kb from the central, usually conserved, region of the genome. ORFs corresponding to fpv121 (cc-chemokine family), fpv122 and fpv123 (VAR B22R family), fpv124 (N1R/p28 family) and fpv125 (V-type Ig domain gene family) are deleted in FeP2 at this site. The five FPVUS genes that are deleted from the central region of FeP2 are all involved in viral host range and virulence, and their loss may indicate that FeP2 is more attenuated than FPVUS. This is supported by the fact that the attenuated FWPV strain, FP9, lacks two of these genes (fpv124 and fpv125) as a result of attenuation (passage)-specific mutations [27].

In addition to this large deletion relative to the FPVUS genome, FeP2 has 36 deleted ORFs, most of which are members of multi-gene families, the disruption of which has been implicated in the attenuation of poxviruses [27]. In light of these ~~-a~~ttenuation"- specific mutations, it is interesting that FeP2 has retained cellular and host range genes, such as C7L (fep223) and IL-10 (fep014). To have been kept intact, these genes must confer some advantage to the virus. The poxvirus C7L family of host range genes functions by mediating poxvirus host range and antagonising the host defence system [332]. VACV C7L orthologues are found in all OPVs and most mammalian poxviruses, with the exception of molluscum contagiosum virus and parapoxviruses [333]. C7L-like genes are not found in the sequenced CNPV or FWPV genomes. IL-10 is a cytokine that has both immunostimulatory and immunosuppressive functions [186], and other pox IL-10 orthologues have been shown to be immunomodulatory in function [189, 190, 331]. Putative orthologues of IL-10 are also found in ORF virus, BPSV [9], LSDV [187] and YLDV [188]. CNPV encodes an IL-10 orthologue with limited similarity to that of FeP2 (22.05% aa identity) and FWPV does not encode an IL-10 gene [25]. The function and benefit of either of these genes in FeP2 is unknown, although it is likely that they would have immunomodulatory function in the infected host.

While it is interesting that FeP2 produces a more inflammatory response on chick CAMs than CNPV and FWPV, this may be a result of a lack of immunomodulatory genes in FeP2 and reduced suppression of the host response to this virus. Several differences between the genomes of FeP2 and the relatively closely related FWPV exist which could account for the differences in phenotype observed on chick CAMs. One possible explanation is that FWPV contains two genes with homology to cellular β-nerve growth factor (β-NGF) (fpv072 and fpv076). FWPV encoded β-NGF is thought to be involved in promoting survival in infected cells and could have a role

in inhibiting antiviral immune responses in the host [25]. FeP2 encodes a severely truncated β-NGF gene homologue, which is unlikely to be functional. The β-NGF genes in FWPV could be responsible for the reduced inflammation observed in FWPV-infected CAMs in comparison to that of FeP2.

It would be beneficial to thoroughly understand the behaviour of any candidate vaccine vector both *in vitro* and *in vivo*. To this end, we examined the *in vitro* growth kinetics and morphogenesis of FeP2 in permissive and non-permissive cells and compared these to other viruses, including MVA, FWPV, CNPV and PEPV. CEF cells were shown to be permissive for FeP2 and the avipoxvirus exhibited complete morphogenesis following a similar sequence of events to other APVs *in vitro* [71]. Conversely, productive growth of FeP2 is inhibited in HeLa cells and morphogenesis was severely restricted. The morphogenesis of FeP2 in Hela cells was similar to the morphogenesis of CNPV observed in mammalian cell lines [271]. Investigation of FeP2 growth in additional human cell lines and foreign gene expression experiments would be useful to determine whether or not this virus could proceed far enough in its life cycle to sufficiently express transgenes within human cells.

Both permissive and non-permissive cells infected with all the viruses analysed (FeP2, MVA, FWPV, CNPV and PEPV) failed to undergo apoptosis (chapter 3). However, for all viruses, CPE was observed (not shown) indicating that the cells are dying by some other process. In mouse spleens, we show that MVA, FWPV and CNPV up regulate caspase 1, which is involved in pyroptosis and not apoptosis. Taken together, these results could indicate that these viruses may induce other programmes of cell death, such as pyroptosis and necrosis, which are not observed by apoptosis-specific assays.

The apoptosis assays performed in chapter 3 do indicate that the five viruses all inhibit apoptosis, since inhibition of camptothecin-induced apoptosis is observed. Poxviruses encode several modulators of apoptosis (reviewed in chapter 1, section 1.4.4) but it is difficult to speculate which gene or genes could be responsible for the inhibition observed. Since all the viruses inhibit apoptosis it is unlikely that either the large internal deletion in the FeP2 genome, nor the insertion of the VACV C7L gene, could be solely responsible for phenotype observed. The observed inhibition of apoptosis could well result from a combination of gene products.

The comparison of host responses produced by different poxvirus vectors would aid in the assessment and rational design of improved vaccines. Host-range restricted

poxviruses have been shown to successfully activate the host immune system. In order to investigate the ability of different poxviruses to interact with the mammalian host, we compared host gene expression profiles in the spleens of BALB/c mice in response to infection (10^5 pfu/mouse). The findings presented here indicate that six, genetically diverse host-restricted poxviruses, CNPV, FWPV, FeP2, PEPV, MVA and LSDV, produce qualitatively and quantitatively distinct host responses in an *in vivo* mouse model.

The differences in host gene expression likely occur through several mechanisms, including viral recognition, tropism and host immune response modulation. Although host-restricted poxviruses are unable to complete their replication cycle in mammalian cells, these viruses are able to express large amounts of their genome, allowing for the expression of immunomodulatory proteins which may shape the host immune response and subsequent gene expression changes in the mouse spleen. It is likely that the addition of a transgene encoding foreign antigen could affect host responses to a poxvirus-vectored vaccine candidate. Zhang et al (2007) showed that the inclusion of a *gag/pro/env* expression cassette into MVA and CNPV altered apoptosis kinetics in infected cells, as compared to cells infected with corresponding empty vectors [136].

Interestingly, South African APVs, FeP2 and PEPV, produced the smallest response in the mouse spleens. Even compared to PEPV, FeP2 infection resulted in remarkably little change in host gene expression. Since APVs are restricted to avian hosts, one would expect to observe fewer differences between the mammalian host responses induced by them compared to those induced by MVA and LSDV. Here we show that three relatively closely related APVs (FWPV, FeP2 and PEPV) induce significant differences in gene expression in the host.

It remains to be determined whether or not an increased vector-specific innate immune response would be beneficial for a poxvirus-based vaccine. Although MVA- and CNPV-vectored vaccine candidates have been shown to be immunogenic in clinical trials [60, 410], these responses have been limited and require further improvement. MVA virus-specific immunogenicity has been shown to be significantly reduced compared to that of VACV Western Reserve (VACV WR) [411]. Host transcriptional responses in HeLa cells following VACV WR infection was markedly different to that observed after MVA infection [87, 90]. Following VACV WR infection, only 37 host genes were upregulated in HeLa Cells, whereas 163 were

upregulated in response to MVA infection [87, 90]. It is possible that the reduced host response to infection correlates positively with the improved immunogenicity of VACV WR compared to MVA, although this has not been directly determined. Alternatively, while MVA is known to induce a poor immune response against itself, it can induce strong immune responses to encoded heterologous antigen [412]. Whether or not host gene expression changes correlate to adaptive responses to heterologous antigen remains to be delineated.

FP9 has been shown to be a more immunogenic recombinant vaccine vector than the Webster FPV-M strain [413]. Passage-specific, attenuation mutations are thought to be responsible for the difference in immunogenicity between these two viruses [414]. Unlike FP9, the genome sequence and passage history of the FWPV-DCEP 25 strain used in this study is unknown. Due to its limited ability to replicate in CEFs (chapter 3), one can assume that it is not an attenuated, or tissue culture-adapted strain of FWPV. Therefore it is possible that the FWPV strain assessed here would not be as immunogenic as FP9; however, without genomic sequence information and further investigation, this is purely speculative.

In another study of FWPV, complete redundancy has been shown for type I IFNs in the generation of adaptive immunity to FWPV-encoded antigen [111]. In addition type I IFNs do not affect the formation of an antibody response to CNPV [415]. While type I IFN responses are important for the induction of the immune response to virus infection, they may decrease the immunogenicity of viral-vectored vaccines by promoting anti-viral responses and subsequent viral clearance [96]. In our study, we show that all viruses analysed, except PEPV and FeP2, induced strong type I IFN responses (chapter 5). It would be interesting to determine what effect the type I IFN responses (or lack thereof) have on the immunogenicity of these viruses.

One could speculate that the inability of FeP2 to induce a response at 24hrs in mice could be related to the apparent inefficiency of FeP2 to enter and proceed to late stages of morphogenesis in HeLa cells. CNPV has been shown to be similarly restricted in human cells [271]. Since this experiment was only performed at one time point, it is possible that the FeP2 (and PEPV) response could occur at an earlier or later time point and was missed at 24hrs. Investigation at further time points is required.

It is of great interest and significance that CNPV and FWPV induce the up regulation of the two immunoglobulin genes Ighg and Ighg3 (IgG3) (chapter 5, Fig. 5.5B). This result supports the finding that the CNPV, ALVAC-HIV (vCP1521) prime component of RV144 may be responsible for stimulating HIV-1–specific IgG3 antibodies which correlated with decreased risk of HIV-1 infection in the RV144 trial. The up regulation of an additional immunoglobulin (Ig) gene, Ig heavy constant mu (Ighm), by CNPV, suggests that this virus may be a good candidate for the induction of an antibody response. Conversely, LSDV, which uniquely down regulates the Ig (light chain) kappa chain complex (IgK) and fails to up regulate any Ig genes, may be a poor inducer of an antibody response. However, several LSDV-based vaccines have been shown to induce vaccine insert-specific antibody responses [338, 397, 398]. Further studies, which directly correlate innate immune signatures with subsequent adaptive responses, are required.

The main aims of this study were to characterise South African APVs and to investigate the potential of one of these viruses for use as a vaccine vector candidate. Although FeP2 failed to induce a significant innate immune response at 24hrs in the mouse spleen, further investigation is required to determine whether an immune response can be induced in response to FeP2-encoded antigen. On one hand, it is possible that this virus simply does not enter mammalian cells sufficiently enough to produce a response. However, morphogenesis of FeP2 in HeLa cells was similar to that of CNPV in human cells [271]. Furthermore, FeP2 was shown to actively inhibit apoptosis in these cells. It would therefore be important to establish the gene expression changes induced by FeP2 infection at different time points and to determine whether or not FeP2 could efficiently express a transgene in mammalian cells, and whether or not the insertion of a transgene would influence the innate immune response *in vivo*. These experiments are underway in our laboratory.

While it is too early to categorically state whether or not FeP2 would make a good candidate vaccine vector we are able to draw the following conclusions: FeP2 can be grown in CEFs, which will allow for the production of a recombinant virus. No evidence of FeP2 entry into the nucleus was observed, in permissive or non-permissive cells, and therefore a strictly cytoplasmic site of replication is concluded. FeP2 also exhibits several aspects of attenuation when compared to other APV strains. Taken together, these characteristics confer a potential significant safety advantage to this virus. In addition, FeP2 exhibits similar growth kinetics and

morphogenesis to CNPV and FWPV in human cells, and all three viruses inhibit apoptosis in these cells to a similar degree. The failure to induce a significant innate immune response *in vivo* is surprising; however, this may be an advantage to a potential vaccine vector as the vector will not be cleared quickly. Further studies to determine what effect this will have on potential vaccine efficacy are required.

Appendix 1

Composition of reagents

1.1 McIlvains Buffer pH 7.4

Solution A: 1.8mM citric acid (2.1g citric acid in 100ml dH_2O)

Solution B: 0.36mM $Na_2HPO_4.12H_2O$ (7.2g $Na_2HPO_4.12H_2O$ in 100ml dH_2O)

Add 18.17ml Solution A and 1.83ml Solution B and make up to 1L with dH_2O.

1.2 10x Phosphate Buffered solution (PBS)

80g NaCl

2.0g KCl

14.4g Na_2HPO_4

2.4g KH_2PO_4

Dilute in 800ml dH_2O. Adjust pH to 7.4 and make up to a volume of 1L with dH_2O.

1x solution made up with 25ml 10X PBS added to 250ml dH_2O

1.3 PBS + Penicillan/Streptomycin and Fungin (PSF)

1x PBS containing the following:

500U/ml penicillin

100µg/ml streptomycin

1µg/ml fungin

1.4 Physiological saline

0.85% w/v NaCl

0.85g NaCl

Made up to a final volume of 100 ml dH_2O

1.5 TE Buffer pH 9.0

10mM Tris-HCL

1mM EDTA

Made up in 400ml dH_2O, adjust pH to 9 and make up to 500ml with dH_2O.

1.6 Lysis buffer

10% N-lauryl sarcosinate

50 mM Tris pH7.8

200 mM β-mercaptoethanol

1.7 1x Tris-borate-EDTA (TBE) buffer

89mM Tris (269g)

89mM Boric Acid (137.6g)

2mM EDTA (18.63g)

Made up to 25L with dH_2O

1.8 10% buffered formalin

37–40 % Formaldehyde

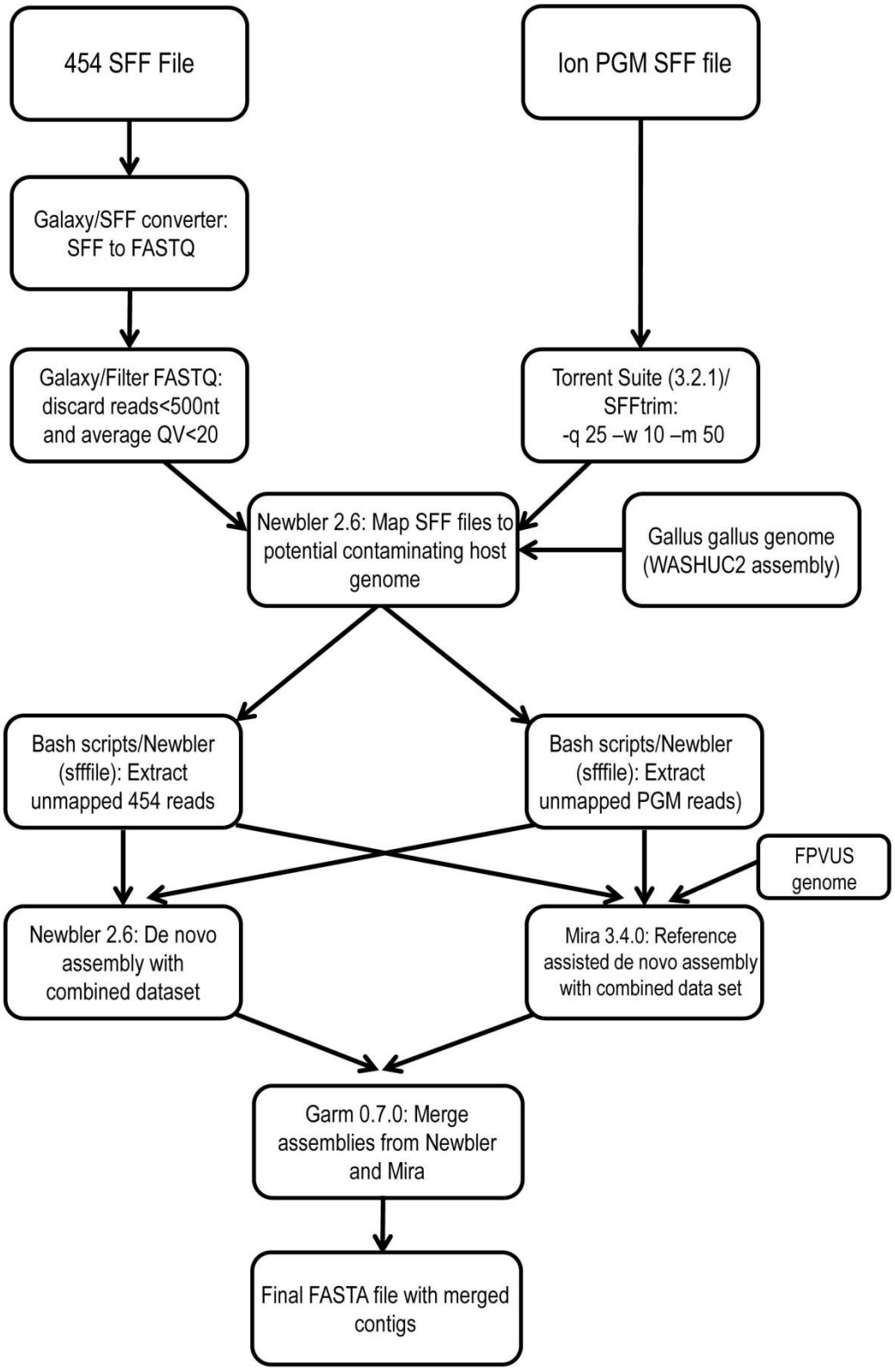
35.03 M NaH2PO4.H20

21.84 M Na2HPO4 (anhydrous)

Made up to 1L with distilled water; pH 7.4

Appendix 2

Customized bioinformatics pipeline for the pre-processing and assembly of pigeonpox virus (FeP2) (Performed by Anelda van der Walt)



Appendix 3

R code used

```
> ##############################################################################
> #R script for analysis of gene expression by poxviruses#
> ##############################################################################

> #set working directory
> setwd("c:/R/data/RawData")
> #view files in working directory
> dir("c:/R/data/RawData")

> #load packages
> library(affy)
> library(limma)
> library(oligo)
> library(annotate)
> library(genefilter)
> library(Biobase)
> library(RColorBrewer)
> library(multtest)
> library(ArrayTools)
> library(pathview)
> library(gage)
> library(GOstats)
> library(ReactomePA)
> library(biomaRt)
> library(GO.db)
> library(mogene20stprobeset.db)
> library(mogene20sttranscriptcluster.db)
> library(genefilter)
> library(gplots)

> #load data
> RawData <-ReadAffy

> #read in .cel files
> RawData <- read.celfiles(list.celfiles("C:/R/data/RawData", full.names = T),verbose=TRUE)

> #load phenotype data
> pd<-read.AnnotatedDataFrame("phenodata.txt",header=TRUE,row.names=1)
> pd.2<-read.table("phenodata.txt",header=T,row.names=1)
> x <- varMetadata(pd)
> x <- data.frame(x, channel = "_ALL_")
> varMetadata(pd) <- x

> #RMA normalisation (background correction, normalizing and expression calculation)
> OligoEset<-rma(RawData,target="core")
> pData(OligoEset)<-pd.2

> #boxplots
> boxplot(OligoEset, col="red",names=pd$label,las=2,main="Normalized data")

> #hist
> hist(OligoEset, fig=TRUE)

> # Scatterplots
> tiff(filename="scatter_OligoEset.tif")
> pairs(exprs(OligoEset)[,1:7], pch=".",main="Scatter plots",col=TRUE)
> dev.off()

> ##############################################################################
> #annotation
> ##############################################################################

> # Which platform?
> OligoEset@annotation
> ls("package:mogene20sttranscriptcluster.db")
> ls("package:mogene20stprobeset.db")

> # Get the transcript cluster IDs from the expressionset
> ID <- featureNames(OligoEset)

> # Look up the Gene Symbol, name, and Ensembl Gene ID for each of those IDs
> Symbol <- getSYMBOL(ID, "mogene20sttranscriptcluster.db")
```

```
> Name <- as.character(lookUp(ID, "mogene20sttranscriptcluster.db", "GENENAME"))
> Ensembl <- as.character(lookUp(ID, "mogene20sttranscriptcluster.db", "ENSEMBL"))
> Entrez <- as.character(lookUp(ID, "mogene20sttranscriptcluster.db", "ENTREZID"))
> #GO<-as.character(lookUp(ID, "mogene20sttranscriptcluster.db", "GO"))

> # Make a temporary data frame with all those identifiers
> tmp <- data.frame(ID=ID, Symbol=Symbol, Name=Name, Ensembl=Ensembl,Entrez=Entrez, stringsAsFactors=F)

> # set the featureData for your expressionset using the data frame you created above.
> fData(OligoEset) <- tmp

> ###############################################################################
> #Non-specific filtering (GeneFilter)
> ###############################################################################

> #intensity filter - (intensity of a gene should be >log2(100) in at least 20% of the samples)

> f1<-pOverA(0.25, log2(100))

> #variance filter - (the interquartile range of log2-intensities should be at least 0.5)
> f2<-function(x)(IQR(x) > 0.5)

> ff<-filterfun(f1,f2)
> selected <- genefilter(OligoEset, ff)
> sum(selected)
> esetSub=OligoEset[selected,]

> ###############################################################################
> #differential expression
> ###############################################################################
> #limma for linear model of data

> # Export all affy expression values to a tab delimited text file
> write.exprs(esetSub, file="OligoEset.txt")

> #differential expression analysis
> # Create appropriate design matrix and assign column names
> design <- model.matrix(~-1+factor(c("EGG","EGG","EGG","ILSDV","ILSDV","ILSDV","MVA","MVA",
  "MVA","FWPV","FWPV","FWPV", "CNPV","CNPV","CNPV","FPK2", "FPK2", "FPK2", "PEPV","PEPV","PEPV")))
> colnames(design)<-c("EGG","ILSDV","MVA","FWPV","CNPV","FPK2","PEPV")

> # Create appropriate contrast matrix for pairwise comparisons
> contrast.matrix <- makeContrasts(EGG-ILSDV, EGG-MVA, EGG-FWPV, EGG-CNPV, EGG-FPK2,EGG-PEPV,
  levels=design)

> # Fit a linear model for each gene based on the given series of arrays
> fit <- lmFit(esetSub, design)

> # Compute estimated coefficients and standard errors for a given set of
> contrasts
> fit2 <- contrasts.fit(fit, contrast.matrix)

> # Compute moderated t-statistics and log-odds of differential expression by empirical Bayes shrinkage of the
  standard errors towards a common value
> fit2 <- eBayes(fit2)

> # Generate list of top 10 DEGs for first comparison
> topTable(fit2, coef=1, adjust="fdr", sort.by="B",p.value=0.05,number=10)

> #Generate heatmap
> list=c()
> for (i in 1:6){
> genes<-topTable(fit2, coef=i, adjust="fdr", sort.by="B",p.value=0.05,number=600)$ID
> list<-c(list,genes)
> }
> list2<-unique(list)
> length(list2)

> gr <- decideTests(fit2 , adjust.method="none")
> gr2 <- decideTests(fit2 , adjust.method="fdr")

> results<-exprs(OligoEset)[list2,]
```

```
> colnames(results)<-pd$label

> heatmap(results,)
> heatmap(results,scale="none")

> #heatmap.2 using gplots
> heatmap.2(results, col=redgreen(75), scale="none", key=TRUE, symkey=FALSE, density.info="none", trace="none",
  cexRow=0.5)

> #generate gene lists (dif. expr. genes) for each comparison (1-6)
> listcom1<-topTable(fit2, coef=1, adjust="fdr", sort.by="B",resort.by="logFC", p.value=0.05,number=Inf)
> write.csv(listcom1, file="listcom1.csv")

> listcom2<-topTable(fit2, coef=2, adjust="fdr", sort.by="B",resort.by="logFC", p.value=0.05,number=Inf)
> write.csv(listcom2, file="listcom2.csv")

> listcom3<-topTable(fit2, coef=3, adjust="fdr", sort.by="B",resort.by="logFC", p.value=0.05,number=Inf)
> write.csv(listcom3, file="listcom3.csv")

> listcom4<-topTable(fit2, coef=4, adjust="fdr", sort.by="B",resort.by="logFC", p.value=0.05,number=Inf)
> write.csv(listcom4, file="listcom4.csv")

> listcom5<-topTable(fit2, coef=5, adjust="fdr", sort.by="B",resort.by="logFC", p.value=0.05,number=Inf)
> write.csv(listcom5, file="listcom5.csv")

> listcom6<-topTable(fit2, coef=6, adjust="fdr", sort.by="B",resort.by="logFC", p.value=0.05,number=Inf)
> write.csv(listcom6, file="listcom6.csv")


> ##############################################################################
> #analysis with cluster profiler

> library(DOSE)
> library(clusterProfiler)
> library(org.Mm.eg.db)
> library(GO.db)
> library(pathview)

> #comparison1 (egg vs iLSDV)

> #data input
> listcom1<-read.csv("c:/R/data/RawData/listcom1.csv")
> head(listcom1)

> # annotate with entrez info
> all_genes1 <- listcom1$Entrez
> all_genes1 <- all_genes1[!is.na(all_genes1)]
> all_genes1<-all_genes1[!duplicated(all_genes1)]
> all_genes1
> LSDV<-as.character(all_genes1)

> #GO classification
> ggo <-groupGO(LSDV, organism="mouse", ont = "BP", level=3, readable = TRUE)
> head(summary(ggo))

> #GO enrichment analysis - HYPERGEOMETRIC MODEL (left out universe="")
> ego<-enrichGO(gene=LSDV,organism="mouse", ont="BP", pvalueCutoff=0.05, readable=TRUE)
> head(summary(ego))

> #GO enrichment analysis - KEGG pathway enrichment analysis
> kk<-enrichKEGG(gene=LSDV,organism="mouse", pvalueCutoff=0.05, readable=TRUE)
> head(summary(kk))

> #visualization of results
> barplot(ggo, drop=TRUE, showCategory=12)
> barplot(ego, drop=TRUE, showCategory=10)
> barplot(kk, drop=TRUE, showCategory=10)
> cnetplot(ego, categorySize="pvalue", foldChange=all_genes1)
> cnetplot(kk, categorySize="geneNum", foldChange=all_genes1)

> # visualisation of pathways with pathview
> require(pathview)
```

```
> setwd("c:/R/data/pathways/lsdv")
> head(summary(kk))

> For each pathway:
> #Type I diabetes mellitus
> mmu04940<-pathview(gene.data=LSDV, pathway.id="mmu04940", species = "mmu", low = list(gene = "green"), mid
  =  list(gene = "gray"), high = list(gene = "red"))

> ##############################################################################################
> #comparison2 (egg vs MVA)

> #data input
> listcom2<-read.csv("c:/R/data/RawData/listcom2.csv")
> head(listcom2)

> # annotate with entrez info
> all_genes2 <- listcom2$Entrez
> all_genes2 <- all_genes2[!is.na(all_genes2)]
> all_genes2<-all_genes2[!duplicated(all_genes2)]
> all_genes2
> MVA<-as.character(all_genes2)

> #GO classification
> ggo2 <-groupGO(MVA, organism="mouse", ont = "BP", level=3, readable = TRUE)
> head(summary(ggo2))

> #GO enrichment analysis - HYPERGEOMETRIC MODEL (left out universe="")
> ego2<-enrichGO(gene=MVA,organism="mouse", ont="BP", pvalueCutoff=0.05, readable=TRUE)
> head(summary(ego2))

> #GO enrichment analysis - KEGG pathway enrichment analysis
> kk2<-enrichKEGG(gene=MVA,organism="mouse", pvalueCutoff=0.05, readable=TRUE)
> head(summary(kk2))

> #visualization of results
> barplot(ggo2, drop=TRUE, showCategory=12)
> barplot(ego2, drop=TRUE, showCategory=10)
> barplot(kk2, drop=TRUE, showCategory=10)
> cnetplot(ego2, categorySize="pvalue", foldChange=all_genes1)
> cnetplot(kk2, categorySize="geneNum", foldChange=all_genes1)

> # visualisation of pathways with pathview
> require(pathview)

> ##############################################################################################
> #comparison3 (egg vs FWPV)

> #data input
> listcom3<-read.csv("c:/R/data/RawData/listcom3.csv")
> head(listcom3)

> # annotate with entrez info
> all_genes3 <- listcom3$Entrez
> all_genes3 <- all_genes3[!is.na(all_genes3)]
> all_genes3<-all_genes3[!duplicated(all_genes3)]
> all_genes3
> FWPV<-as.character(all_genes3)

> #GO classification
> ggo3 <-groupGO(FWPV, organism="mouse", ont = "BP", level=3, readable = TRUE)
> head(summary(ggo3))

> #GO enrichment analysis - HYPERGEOMETRIC MODEL (left out universe="")
> ego3 <-enrichGO(gene=FWPV,organism="mouse", ont="BP", pvalueCutoff=0.05, readable=TRUE)
> head(summary(ego3))

> #GO enrichment analysis - KEGG pathway enrichment analysis
> kk3 <-enrichKEGG(gene=FWPV,organism="mouse", pvalueCutoff=0.05, readable=TRUE)
> head(summary(kk3))

> #visualization of results
> barplot(ggo3, drop=TRUE, showCategory=12)
```

```
> barplot(ego3, drop=TRUE, showCategory=10)
> barplot(kk3, drop=TRUE, showCategory=10)
> cnetplot(ego3, categorySize="pvalue", foldChange=all_genes1)
> cnetplot(kk3, categorySize="geneNum", foldChange=all_genes1)

> # visualisation of pathways with pathview
> require(pathview)

> ##########################################################################################
> #comparison4 (egg vs CNPV)

> #data input
> listcom4<-read.csv("c:/R/data/RawData/listcom4.csv")
> head(listcom4)

> # annotate with entrez info
> all_genes4 <- listcom4$Entrez
> all_genes4 <- all_genes4[!is.na(all_genes4)]
> all_genes4<-all_genes4[!duplicated(all_genes4)]
> all_genes4
> CNPV<-as.character(all_genes4)

> #GO classification
> ggo4 <-groupGO(CNPV, organism="mouse", ont = "BP", level=3, readable = TRUE)
> head(summary(ggo4))

> #GO enrichment analysis - HYPERGEOMETRIC MODEL (left out universe="")
> ego4<-enrichGO(gene=CNPV,organism="mouse", ont="BP", pvalueCutoff=0.05, readable=TRUE)
> head(summary(ego4))

> #GO enrichment analysis - KEGG pathway enrichment analysis
> kk4<-enrichKEGG(gene=CNPV,organism="mouse", pvalueCutoff=0.05, readable=TRUE)
> head(summary(kk4))

> #visualization of results
> barplot(ggo4, drop=TRUE, showCategory=12)
> barplot(ego4, drop=TRUE, showCategory=10)
> barplot(kk4, drop=TRUE, showCategory=10)
> cnetplot(ego4, categorySize="pvalue", foldChange=all_genes1)
> cnetplot(kk4, categorySize="geneNum", foldChange=all_genes1)

> # visualisation of pathways with pathview
> require(pathview)

> ##########################################################################################
> #comparison5 (egg vs FeP2)

> #data input
> listcom5<-read.csv("c:/R/data/RawData/listcom5.csv")
> head(listcom5)

> # annotate with entrez info
> all_genes5 <- listcom5$Entrez
> all_genes5 <- all_genes5[!is.na(all_genes5)]
> all_genes5<-all_genes5[!duplicated(all_genes5)]
> all_genes5
> FeP2<-as.character(all_genes5)

> #GO classification
> ggo5 <-groupGO(FeP2, organism="mouse", ont = "BP", level=3, readable = TRUE)
> head(summary(ggo5))

> #GO enrichment analysis - HYPERGEOMETRIC MODEL (left out universe="")
> ego5<-enrichGO(gene=FeP2,organism="mouse", ont="BP", pvalueCutoff=0.05, readable=TRUE)
> head(summary(ego5))

> #GO enrichment analysis - KEGG pathway enrichment analysis
> kk5<-enrichKEGG(gene=FeP2,organism="mouse", pvalueCutoff=0.05, readable=TRUE)
> head(summary(kk5))

> #visualization of results
> barplot(ggo5, drop=TRUE, showCategory=12)
```

```
> barplot(ego5, drop=TRUE, showCategory=10)
> barplot(kk5, drop=TRUE, showCategory=10)
> cnetplot(ego5, categorySize="pvalue", foldChange=all_genes1)
> cnetplot(kk5, categorySize="geneNum", foldChange=all_genes1)

> # visualisation of pathways with pathview
> require(pathview)

> ##########################################################################################
> #comparison6 (egg vs PEPV)

> #data input
> listcom6<-read.csv("c:/R/data/RawData/listcom6.csv")
> head(listcom6)

> # annotate with entrez info
> all_genes6 <- listcom6$Entrez
> all_genes6 <- all_genes6[!is.na(all_genes6)]
> all_genes6<-all_genes6[!duplicated(all_genes6)]
> all_genes6
> PEPV<-as.character(all_genes6)

> #GO classification
> ggo6 <-groupGO(PEPV, organism="mouse", ont = "BP", level=3, readable = TRUE)
> head(summary(ggo6))

> #GO enrichment analysis - HYPERGEOMETRIC MODEL (left out universe="")
> ego6<-enrichGO(gene=PEPV,organism="mouse", ont="BP", pvalueCutoff=0.05, readable=TRUE)
> head(summary(ego6))

> #GO enrichment analysis - KEGG pathway enrichment analysis
> kk6<-enrichKEGG(gene=PEPV,organism="mouse", pvalueCutoff=0.05, readable=TRUE)
> head(summary(kk6))

> #visualization of results
> barplot(ggo6, drop=TRUE, showCategory=12)
> barplot(ego6, drop=TRUE, showCategory=10)
> barplot(kk6, drop=TRUE, showCategory=10)
> cnetplot(ego6, categorySize="pvalue", foldChange=all_genes1)
> cnetplot(kk6, categorySize="geneNum", foldChange=all_genes1)


> ##########################################################################################
> #Biological Theme Comparison

> allcomparison<-cbind(LSDV,MVA,FWPV,CNPV,FeP2,PEPV)
> allcomparison
> write.csv(allcomparison, file="c:/R/data/RawData/allc.csv")

> allc<-read.csv("c:/R/data/RawData/allc.csv") #removed first column of numbers 1-.. in excel
> names(allc)
> allc[1]

> ck<-compareCluster(geneCluster=allc, fun="enrichKEGG", organism="mouse", pvalueCutoff=0.05, readable=TRUE)
> head(summary(ck))
> cGO<-compareCluster(geneCluster=allc, fun="enrichGO",ont="BP", organism="mouse", pvalueCutoff=0.05,
  readable=TRUE)
> cGOMF<-compareCluster(geneCluster=allc, fun="enrichGO",ont="MF", organism="mouse", pvalueCutoff=0.05,
  readable=TRUE)
> cGOCC<-compareCluster(geneCluster=allc, fun="enrichGO",ont="CC", organism="mouse", pvalueCutoff=0.05,
  readable=TRUE)

> plot(ck, type = "dot", showCategory=NULL, by = "count", title = "KEGG enrichment", font.size = 12)
> plot(ck, type = "bar", showCategory=NULL, by = "count", title = "KEGG enrichment", font.size = 12)
> plot(ck, type = "bar", showCategory=NULL, by = "percentage", title = "KEGG enrichment", font.size = 12)

> plot(cGO, type = "dot", showCategory=30, by = "count", title = "GO enrichment", font.size = 12)
> plot(cGO, type = "bar", showCategory=30, by = "count", title = "GO enrichment", font.size = 12)

> plot(cGOMF, type = "dot", showCategory=20, by = "count", title = "GO enrichment", font.size = 12)
> plot(cGOCC, type = "dot", showCategory=20, by = "count", title = "GO enrichment", font.size = 12)
```

Appendix 4

Full lists of up- and down regulated genes in mouse spleens in response to modified vaccinia Ankara (MVA), lumpy skin disease virus (LSDV), canarypox virus (CNPV), fowlpox virus (FWPV), pigeonpox (FeP2) and penguinpox virus (PEPV).

Table a1. Full list of annotated up regulated genes in mouse spleens in response to CNPV, FeP2, FWPV, LSDV, MVA and PEPV. Differences in Log_2 Fold Changes (between each virus and the control) are depicted.

Symbol	Name	Entrez	MVA	LSDV	CNPV	FWPV	PEPV	FeP2
1500012F01Rik	RIKEN cDNA 1500012F01 gene	68949	1.2	1.1	1.5	1.6	-	-
1500012F01Rik	RIKEN cDNA 1500012F01 gene	68949	-	1	-	-	-	-
2610524H06Rik	RIKEN cDNA 2610524H06 gene	330173	-	-	1.1	1.2	-	-
3110062M04Rik	RIKEN cDNA 3110062M04 gene	78412	1.2	1.7	1.5	1.4	-	-
3830406C13Rik	RIKEN cDNA 3830406C13 gene	218734	-	-	1	-	-	-
4933412E12Rik	RIKEN cDNA 4933412E12 gene	71086	-	1	-	1	-	-
9330175E14Rik	RIKEN cDNA 9330175E14 gene	320377	-	1.1	-	1.1	-	-
A530040E14Rik	RIKEN cDNA A530040E14 gene	621875	1.1	-	-	-	-	-
A530064D06Rik	RIKEN cDNA A530064D06 gene	328830	2	1.8	2.2	1.9	1.2	-
Abracl	ABRA C-terminal like	73112	-	-	-	1	-	-
Abtb2	ankyrin repeat and BTB (POZ) domain containing 2	99382	-	1.1	-	1.1	-	-
Adamts1	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1	11504	-	1.1	-	-	-	-
Adar	adenosine deaminase, RNA-specific	56417	-	1	-	-	-	-
AI607873	expressed sequence AI607873	226691	1.8	2	1.8	1.6	1.1	-
Aif1	allograft inflammatory factor 1	11629	-	-	-	1.1	-	-
Akr1b10	aldo-keto reductase family 1, member B10 (aldose reductase)	67861	-	-	1.1	1.1	-	-
Alox12	arachidonate 12-lipoxygenase	11684	-	-	1	1.2	-	-
Alpl	alkaline phosphatase, liver/bone/kidney	11647	1.3	1.3	1	1.2	-	-
Amica1	adhesion molecule, interacts with CXADR antigen 1	270152	-	1.1	-	1.1	-	-
Angptl4	angiopoietin-like 4	57875	1.1	1.5	-	1.6	1.1	-
Anxa1	annexin A1	16952	-	-	1.6	1.8	-	-
Anxa2	annexin A2	12306	-	-	-	1.1	-	-
Anxa4	annexin A4	11746	1.8	1.9	1.7	2.1	1.1	-
Aoah	acyloxyacyl hydrolase	27052	-	-	1.1	-	-	-
Apobec1	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1	11810	-	-	1.2	1.2	-	-
Apod	apolipoprotein D	11815	1.5	1.3	1.5	1.4	-	-
Apol9b	apolipoprotein L 9b	71898	2.3	2.7	2.6	2.4	-	-
Asb13	ankyrin repeat and SOCS box-containing 13	142688	1.2	1	1.1	1.3	-	-
Asprv1	aspartic peptidase, retroviral-like 1	67855	1.2	1.1	1.2	1.3	-	-
Atf3	activating transcription factor 3	11910	1.3	1.4	1.2	1.4	1.1	-
Atp10a	ATPase, class V, type 10A	11982	1.1	1.2	-	1.1	-	-
Atp8b1	ATPase, class I, type 8B, member 1	54670	1	1.3	1	1.3	-	-
Atp8b4	ATPase, class I, type 8B, member 4	241633	-	1.4	-	-	-	-
AW011738	expressed sequence AW011738	100382	1.1	1.7	1	1	-	-
AW112010	expressed sequence AW112010	107350	2	1.8	1.4	1.8	-	-
B430306N03Rik	RIKEN cDNA B430306N03 gene	320148	1.2	1.4	1.3	1.5	-	-
B4galt4	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 4	56375	-	1.1	-	-	-	-
B4galt5	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5	56336	-	1	-	-	-	-
BC023105	cDNA sequence BC023105	667597	1.3	1.5	1.3	1.4	1.1	-
BC094916	cDNA sequence BC094916	545384	1.2	1.8	-	-	-	-
BC147527	cDNA sequence BC147527	625360	1.2	1.5	-	-	-	-
Bst2	bone marrow stromal cell antigen 2	69550	2.3	3	2.1	2.2	-	-
C1qa	complement component 1, q subcomponent, alpha polypeptide	12259	-	1.1	1	-	-	-
C2	complement component 2 (within H-2S)	12263	1.5	1.8	1.7	1.6	1	-
Cacnb3	calcium channel, voltage-dependent, beta 3 subunit	12297	-	1.2	-	1.1	-	-
Car13	carbonic anhydrase 13	71934	1	1.2	1.1	1.1	-	-
Casp1	caspase 1	12362	1.1	-	1.2	1.1	-	-
Casp4	caspase 4, apoptosis-related cysteine peptidase	12363	1.9	1.6	1.9	2	-	-
Ccdc53	coiled-coil domain containing 53	67282	-	-	-	1	-	-

Ccl2	chemokine (C-C motif) ligand 2	20296	3.5	3.3	2.9	3.3	2.8	-
Ccl3	chemokine (C-C motif) ligand 3	20302	2	2.1	2.3	2	1.4	-
Ccl6	chemokine (C-C motif) ligand 6	20305	-	-	1.3	1.3	-	-
Ccl7	chemokine (C-C motif) ligand 7	20306	3	2.9	2.6	2.9	2.7	1.5
Ccr5	chemokine (C-C motif) receptor 5	12774	1.1	1.5	1.3	1.3	-	-
Ccrl2	chemokine (C-C motif) receptor-like 2	54199	-	1.5	1.2	1.4	-	-
Cd274	CD274 antigen	60533	1.9	2.3	1.6	2.1	1.2	-
Cd5l	CD5 antigen-like	11801	1.2	1.5	1.4	1.1	-	-
Cd69	CD69 antigen	12515	1.8	1.8	1.5	1.7	-	-
Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)	12575	1.7	2	1.5	2	1.2	-
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	12608	-	1	-	-	-	-
Cfb	complement factor B	14962	2.2	2.6	1.6	2	1	-
Chi3l3	chitinase 3-like 3	12655	-	1.2	1.4	1.6	-	-
Chic1	cysteine-rich hydrophobic domain 1	12212	1.1	1.4	1	-	-	-
Clec4a2	C-type lectin domain family 4, member a2	26888	1.2	-	1.5	1.1	-	-
Cmpk2	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	22169	1.3	2	1.3	1.3	-	-
Cndp2	CNDP dipeptidase 2 (metallopeptidase M20 family)	66054	1.2	1.4	1.1	1.4	-	-
Col4a2	collagen, type IV, alpha 2	12827	-	1.1	-	-	-	-
Cp	ceruloplasmin	12870	1.6	1.7	1.6	1.8	1.2	-
Csf2rb2	colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage)	12984	-	1.5	-	1.7	-	-
Csprs	component of Sp100-rs	114564	1.3	-	-	-	-	-
Csprs	component of Sp100-rs	114564	1.5	1.2	-	-	-	-
Csprs	component of Sp100-rs	114564	1.5	1.2	-	-	-	-
Cst7	cystatin F (leukocystatin)	13011	1.2	1.4	1.1	1.4	-	-
Cstb	cystatin B	13014	1.4	1	1.5	1.5	-	-
Ctla2a	cytotoxic T lymphocyte-associated protein 2 alpha	13024	1.1	1	1.1	1.3	-	1.4
Ctsc	cathepsin C	13032	1.1	1.1	-	1.1	-	-
Ctsl	cathepsin L	13039	-	-	1.1	-	-	-
Cxcl10	chemokine (C-X-C motif) ligand 10	15945	2.7	3	2.2	2.8	1.7	-
Cxcl11	chemokine (C-X-C motif) ligand 11	56066	4.5	4.4	3.4	4.3	1.5	-
Cxcl9	chemokine (C-X-C motif) ligand 9	17329	2.1	2.3	-	1.8	-	-
Cycs	cytochrome c, somatic	13063	-	1.1	-	1.1	-	-
Cycs	cytochrome c, somatic	13063	-	1.1	-	1.1	-	-
Cycs	cytochrome c, somatic	13063	-	1.2	-	1.2	-	-
Daxx	Fas death domain-associated protein	13163	2	2.3	1.7	1.9	-	-
Dcn	decorin	13179	-	-	-	1.5	-	-
Ddit4	DNA-damage-inducible transcript 4	74747	1.2	1.2	1.8	1.3	1.4	-
Ddx18	DEAD (Asp-Glu-Ala-Asp) box polypeptide 18	66942	-	-	-	1.1	-	-
Ddx58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	230073	-	1.3	-	-	-	-
Ddx60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	234311	1.7	2.6	1.5	1.4	-	-
Dhx58	DEXH (Asp-Glu-X-His) box polypeptide 58	80861	1.7	2.5	1.6	1.5	-	-
Dram1	DNA-damage regulated autophagy modulator 1	71712	1.2	1.4	-	1.3	-	-
Dtx3l	deltex 3-like (Drosophila)	209200	1	1.5	-	-	-	-
Dusp28	dual specificity phosphatase 28	67446	-	1	-	-	-	-
Ear2	eosinophil-associated, ribonuclease A family, member 2	13587	-	-	1.3	1.5	-	-
Eif2ak2	eukaryotic translation initiation factor 2-alpha kinase 2	19106	1.1	2.1	1.1	1.1	-	-
Emp1	epithelial membrane protein 1	13730	-	-	-	1.1	-	-
Epsti1	epithelial stromal interaction 1 (breast)	108670	-	1.4	-	-	-	-
F830016B08Rik	RIKEN cDNA F830016B08 gene	240328	1.5	2.5	1.9	1.9	1.2	-
Fam136a	family with sequence similarity 136, member A	66488	-	1.1	-	1.3	-	-
Fam26f	family with sequence similarity 26, member F	215900	1.5	1.9	1.1	1.8	-	-
Fbxw17	F-box and WD-40 domain protein 17	109082	-	1.2	-	1	-	-
Fcgr1	Fc receptor, IgG, high affinity I	14129	2.4	2.6	2.4	2.6	1.4	-
Fcgr4	Fc receptor, IgG, low affinity IV	246256	2.5	3.6	2.8	2.9	1.8	-
Fgl2	fibrinogen-like protein 2	14190	-	-	-	1.2	-	-
Folh1	folate hydrolase 1	53320	3.1	2.7	3	2.8	2.1	1.9
Fpr1	formyl peptide receptor 1	14293	1.3	1.1	1.4	1.4	-	-
Fpr2	formyl peptide receptor 2	14289	1.8	1.5	1.7	1.9	-	-
Fscn1	fascin homolog 1, actin bundling protein (Strongylocentrotus purpuratus)	14086	1.2	1.6	-	1	-	-
G530011O06Rik	RIKEN cDNA G530011O06 gene	654820	2.5	1.7	1.8	-	-	-
Gadd45b	growth arrest and DNA-damage-inducible 45 beta	17873	1.3	1.5	1.2	1.5	-	-
Gatm	glycine amidinotransferase (L-arginine:glycine amidinotransferase)	67092	1.2	1.3	1.3	1.4	-	-
Gbp1	guanylate binding protein 1	14468	2.1	2.7	1.6	2.5	-	-
Gbp10	guanylate-binding protein 10	626578	2.1	3.2	1.3	1.9	-	-
Gbp11	guanylate binding protein 11	634650	3.9	4.5	2.9	4.2	1.6	-
Gbp2	guanylate binding protein 2	14469	2.3	2.8	1.5	2.7	-	-
Gbp3	guanylate binding protein 3	55932	1.6	1.8	1.2	1.6	-	-
Gbp4	guanylate binding protein 4	17472	2.1	2.7	1.3	2.4	-	-

Gbp5	guanylate binding protein 5	229898	2	2.8	1.3	2.2	1.1	-
Gbp7	guanylate binding protein 7	229900	1.6	2.2	1.2	1.7	-	-
Gbp8	guanylate-binding protein 8	76074	1.3	1.4	1.4	2.1	-	-
Gbp9	guanylate-binding protein 9	236573	1.2	1.9	1	1.3	-	-
Gca	grancalcin	227960	1.2	1.3	1.4	1.6	-	-
Gdap10	ganglioside-induced differentiation-associated-protein 10	100504486	1	1.6	-	1	-	-
Gla	galactosidase, alpha	11605	1.6	1.8	1.6	1.7	1.1	-
Glipr2	GLI pathogenesis-related 2	384009	1.4	1.5	1.1	1.5	-	-
Gm10495	predicted gene 10495	100504611	-	-	-	1.3	-	-
Gm11772	predicted gene 11772	100503912	1.6	2.2	1.7	1.7	-	-
Gm12185	predicted gene 12185	620913	2	2.6	1.3	2.1	-	-
Gm12250	predicted gene 12250	631323	1.7	2.9	1.1	1.8	-	-
Gm13157	predicted gene 13157	100041677	-	-	-	1.1	-	-
Gm14446	predicted gene 14446	667373	3.3	3.4	2.3	2.5	-	-
Gm15056	predicted gene 15056	100504014	3.8	3.8	3.4	3.7	2.5	1.6
Gm17757	GTPase, very large interferon inducible 1 pseudogene	100417829	-	1.4	-	-	-	-
Gm17757	GTPase, very large interferon inducible 1 pseudogene	100417829	-	1.5	-	-	-	-
Gm1966	predicted gene 1966	434223	1	1.5	-	-	-	-
Gm19705	predicted gene, 19705	100503460	1.5	1.4	1.3	1.5	-	-
Gm19763	predicted gene, 19763	100503548	-	-	-	1	-	-
Gm20134	predicted gene, 20134	100504251	1.7	1.5	1.2	1.3	-	-
Gm20559	predicted gene, 20559	330256	-	1.1	-	-	-	-
Gm4070	predicted gene 4070	100042856	-	1.5	-	-	-	-
Gm4841	predicted gene 4841	225594	1.8	2.1	2	2.1	2	-
Gm4841	predicted gene 4841	225594	2.5	3	2.4	2.8	2.3	-
Gm4951	predicted gene 4951	240327	1.5	2.7	1.3	1.5	-	-
Gm5431	predicted gene 5431	432555	2.6	3.1	2.4	2.3	-	-
Gm6548	eukaryotic translation elongation factor 1 alpha 1 pseudogene	625054	-	1.4	-	-	-	-
Gm6904	predicted gene 6904	628693	1.4	1.9	-	-	-	-
Gm7609	predicted pseudogene 7609	665378	1.4	1.2	-	-	-	-
Gm7609	predicted pseudogene 7609	665378	-	1.5	-	-	-	-
Gm8979	very large inducible GTPase 1 pseudogene	668108	-	1.1	-	-	-	-
Gm8989	very large inducible GTPase 1 pseudogene	668128	-	1.1	-	-	-	-
Gm8995	predicted gene 8995	668139	-	1.1	-	-	-	-
Gmppb	GDP-mannose pyrophosphorylase B	331026	-	1.1	-	-	-	-
Gnb4	guanine nucleotide binding protein (G protein), beta 4	14696	-	1.2	-	-	-	-
Gng12	guanine nucleotide binding protein (G protein), gamma 12	14701	-	-	-	1.1	-	-
Gnl3	guanine nucleotide binding protein-like 3 (nucleolar)	30877	-	-	-	1.1	-	-
Gnpnat1	glucosamine-phosphate N-acetyltransferase 1	54342	1.1	-	-	1	-	-
Gp49a	glycoprotein 49 A	14727	2.6	2.4	2.7	2.7	1.9	2
Gpr128	G protein-coupled receptor 128	239853	1.2	1.4	-	1.2	-	-
Grn	granulin	14824	-	1.1	-	-	-	-
Gsdmd	gasdermin D	69146	-	-	-	1.2	-	-
Gvin1	GTPase, very large interferon inducible 1	74558	-	1.5	-	-	-	-
Gzma	granzyme A	14938	1.8	1.8	2.2	2	-	-
Gzmb	granzyme B	14939	3.7	4.7	4.1	4.2	2.4	-
H2-Q4	histocompatibility 2, Q region locus 4	15015	1	1.2	-	1.2	-	-
H2-Q6	histocompatibility 2, Q region locus 6	110557	1	1	-	1.1	-	-
H2-T22	histocompatibility 2, T region locus 22	15039	1	1.3	-	-	-	-
H2-T23	histocompatibility 2, T region locus 23	15040	1	1.3	-	1	-	-
H2-T24	histocompatibility 2, T region locus 24	15042	-	1.2	-	-	-	-
Hdc	histidine decarboxylase	15186	-	1.4	1.2	1.5	-	-
Helz2	helicase with zinc finger 2, transcriptional coactivator	229003	1.1	1.5	-	-	-	-
Herc6	hect domain and RLD 6	67138	1.3	1.9	1.1	1.1	-	-
Hk3	hexokinase 3	212032	1.5	2	1.7	2	1.2	-
Hmgcs2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	15360	1.3	1.1	2.1	1.7	1.8	2.2
Hnrnph2	heterogeneous nuclear ribonucleoprotein H2	56258	-	-	-	1.1	-	-
Hp	haptoglobin	15439	1.2	1.8	1.7	1.9	1.3	1.3

Hpse	heparanase	15442	-	1.1	-	-	-	-
Hsbp1	heat shock factor binding protein 1	68196	-	-	1.2	1.2	-	-
Hsh2d	hematopoietic SH2 domain containing	209488	1.1	1.4	-	-	-	-
Hspa1b	heat shock protein 1B	15511	2.3	2.9	2.4	2.5	-	-
Ifi202b	interferon activated gene 202B	26388	2.4	2.6	2.1	2.2	-	-
Ifi203	interferon activated gene 203	15950	-	1.1	-	-	-	-
Ifi204	interferon activated gene 204	15951	3.3	4	3.2	3.9	-	-
Ifi205	interferon activated gene 205	226695	1.4	-	1.6	1.8	-	-
Ifi27l2a	interferon, alpha-inducible protein 27 like 2A	76933	-	1.9	-	-	-	-
Ifi35	interferon-induced protein 35	70110	1.1	1.5	1.1	1.2	-	-
Ifi44	interferon-induced protein 44	99899	2	2.5	1.8	1.6	-	-
Ifi44l	interferon-induced protein 44 like	15061	2.1	2.9	2	2	-	-
Ifih1	interferon induced with helicase C domain 1	71586	1.3	2.1	1.2	1.2	-	-
Ifit1	interferon-induced protein with tetratricopeptide repeats 1	15957	2.2	3.3	2	1.7	-	-
Ifit2	interferon-induced protein with tetratricopeptide repeats 2	15958	1.8	2.5	1.8	1.7	-	-
Ifitm3	interferon induced transmembrane protein 3	66141	-	1.4	-	-	-	-
Ifitm6	interferon induced transmembrane protein 6	213002	1.8	1.9	2.4	2.3	1.6	1.5
Ighg	Immunoglobulin heavy chain (gamma polypeptide)	380794	-	-	1.5	1.6	-	-
Ighg3	Immunoglobulin heavy constant gamma 3	380795	-	-	1.3	1.2	-	-
Ighm	immunoglobulin heavy constant mu	16019	-	-	1.1	-	-	-
Igtp	interferon gamma induced GTPase	16145	1.4	2.2	1	1.8	-	-
Iigp1	interferon inducible GTPase 1	60440	1.7	2.9	1.2	1.9	-	-
Il12rb1	interleukin 12 receptor, beta 1	16161	2	2.4	1.7	2.1	1.1	-
Il12rb2	interleukin 12 receptor, beta 2	16162	1.2	1.3	1.3	1.3	-	-
Il15	interleukin 15	16168	1.1	1.3	1.2	1	-	-
Il15ra	interleukin 15 receptor, alpha chain	16169	1.8	1.9	1.5	1.8	1.2	-
Il18bp	interleukin 18 binding protein	16068	1.2	1.7	1.3	1.5	-	-
Il1a	interleukin 1 alpha	16175	1.9	2	2.2	2.3	1.8	-
Il1f9	interleukin 1 family, member 9	215257	1.3	1.6	1.7	1.8	1.2	1
Il1rn	interleukin 1 receptor antagonist	16181	1.3	1.1	-	1.2	-	-
Il2ra	interleukin 2 receptor, alpha chain	16184	1.1	1.2	-	1.3	1.1	-
Il33	interleukin 33	77125	-	1	-	1.1	-	-
Irf1	interferon regulatory factor 1	16362	-	1.3	-	1.1	-	-
Irf7	interferon regulatory factor 7	54123	1.7	2.9	1.7	1.1	-	-
Irg1	immunoresponsive gene 1	16365	2.6	2.5	1.9	2.5	1.6	-
Irgm1	immunity-related GTPase family M member 1	15944	1.4	2.3	1.1	1.4	-	-
Irgm2	immunity-related GTPase family M member 2	54396	1	1.7	-	1.2	-	-
Isg15	ISG15 ubiquitin-like modifier	100038882	1.6	2.2	1.5	1.4	-	-
Jhdm1d	jumonji C domain-containing histone demethylase 1 homolog D (S. cerevisiae)	338523	1.1	1.6	1.1	1.1	-	-
Kcne4	potassium voltage-gated channel, Isk-related subfamily, gene 4	57814	1.2	1.4	0	1.3	-	-
Klk1	kallikrein 1	16612	-	-	1.3	1	-	-
Klrk1	killer cell lectin-like receptor subfamily K, member 1	27007	1.5	1.7	1.6	1.5	-	-
Lap3	leucine aminopeptidase 3	66988	1.1	1.5	1	1.5	-	-
Lcn2	lipocalin 2	16819	1.4	1.6	2.2	2.2	1.4	1.7
Lgals3bp	lectin, galactoside-binding, soluble, 3 binding protein	19039	1.2	1.7	1.1	-	-	-
Lgals3bp	lectin, galactoside-binding, soluble, 3 binding protein	19039	-	1.5	-	-	-	-
Lgals9	lectin, galactose binding, soluble 9	16859	1.2	1.6	1.1	1.2	-	-
Lgmn	legumain	19141	1.3	1.2	1.4	1.4	-	-
Lilrb3	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	18733	-	-	1.1	1	-	-
Lilrb4	leukocyte immunoglobulin-like receptor, subfamily B, member 4	14728	1.6	1.5	1.7	1.7	1.1	-
Lipg	lipase, endothelial	16891	2.4	2.8	2.4	2.5	1.8	1.5
Lrrc16a	leucine rich repeat containing 16A	68732	-	1.2	-	-	-	-
Lrrc61	leucine rich repeat containing 61	243371	1.8	1.4	1.8	2.1	1.3	-
Ltv1	LTV1 homolog (S. cerevisiae)	353258	-	-	-	1.1	-	-
Ly6a	lymphocyte antigen 6 complex, locus A	110454	1.7	1.9	1.2	1.3	-	-
Ly6c1	lymphocyte antigen 6 complex, locus C1	17067	1.8	2.2	1.9	1.9	-	-
Ly6c2	lymphocyte antigen 6 complex, locus C2	100041546	1.3	1.6	1.3	1.1	-	-
Ly6g	lymphocyte antigen 6 complex, locus G	546644	-	1.7	2.5	2.2	-	-
Ly6i	lymphocyte antigen 6 complex, locus I	57248	-	1	-	-	-	-
Ly96	lymphocyte antigen 96	17087	-	-	1.2	-	-	-
Lyrm2	LYR motif containing 2	108755	-	-	-	1	-	-
Marco	macrophage receptor with collagenous structure	17167	-	-	1	-	1.5	1.5
Mcee	methylmalonyl CoA epimerase	73724	-	-	1.1	-	-	-
Med10	mediator of RNA polymerase II transcription,	28077	-	-	-	1.1	-	-

	subunit 10 homolog (NUT2, S. cerevisiae)							
Med11	mediator of RNA polymerase II transcription, subunit 11 homolog (S. cerevisiae)	66172	-	-	-	1.1	-	-
Med4	mediator of RNA polymerase II transcription, subunit 4 homolog (yeast)	67381	-	-	-	1	-	-
Mid1	midline 1	17318	1.3	1.4	-	-	-	-
Mif	macrophage migration inhibitory factor	17319	-	1.1	-	-	-	-
Mir1949	microRNA 1949	100316700	-	1.2	-	-	-	-
Mitd1	MIT, microtubule interacting and transport, domain containing 1	69028	1.2	1.4	1.1	1.2	-	-
Mki67ip	Mki67 (FHA domain) interacting nucleolar phosphoprotein	67949	-	-	0	1.2	-	-
Mlkl	mixed lineage kinase domain-like	74568	2.1	2.7	2	2.3	-	-
Mmp13	matrix metallopeptidase 13	17386	2.7	2.6	2.4	2.4	1.5	-
Mmp19	matrix metallopeptidase 19	58223	1.9	2	2	2	1.5	1.2
Mmp25	matrix metallopeptidase 25	240047	-	1	-	1.1	-	-
Mmp8	matrix metallopeptidase 8	17394	2.7	3	3.1	3.4	2.5	2.8
Mnda	myeloid cell nuclear differentiation antigen	381308	1.8	1.8	1.6	1.7	-	-
Mndal	myeloid nuclear differentiation antigen like	100040462	1.1	1.3	1.1	1.2	-	-
Mov10	Moloney leukemia virus 10	17454	1.1	1.5	-	-	-	-
Mreg	melanoregulin	381269	-	1.1	-	1.1	-	-
Mrpl42	mitochondrial ribosomal protein L42	67270	-	-	1.1	1.2	-	-
Mrpl54	mitochondrial ribosomal protein L54	66047	-	1	-	-	-	-
Ms4a4a	membrane-spanning 4-domains, subfamily A, member 4A	666907	2.6	2.7	2.6	2.5	1.5	1.1
Ms4a4c	membrane-spanning 4-domains, subfamily A, member 4C	64380	1.2	1.5	1.2	1	-	-
Ms4a4d	membrane-spanning 4-domains, subfamily A, member 4D	66607	1.6	1.5	1.6	1.8	-	-
Ms4a6c	membrane-spanning 4-domains, subfamily A, member 6C	73656	1.1	1.1	1.2	1.2	-	-
Ms4a6d	membrane-spanning 4-domains, subfamily A, member 6D	68774	3.2	3.5	3.1	3.3	-	-
Ms4a7	membrane-spanning 4-domains, subfamily A, member 7	109225	1.7	1.4	2	1.9	1.4	-
Msr1	macrophage scavenger receptor 1	20288	2.3	2	2	2.2	1.4	1.1
Mvp	major vault protein	78388	-	1.3	-	1	-	-
Mx1	myxovirus (influenza virus) resistance 1	17857	3.2	3.9	3	2.8	-	-
Mx2	myxovirus (influenza virus) resistance 2	17858	2.7	3.7	2.1	2.2	-	-
Myc	myelocytomatosis oncogene	17869	-	1.1	-	-	-	-
Myd88	myeloid differentiation primary response gene 88	17874	-	-	-	1.2	-	-
N4bp1	NEDD4 binding protein 1	80750	-	1	-	-	-	-
Naa25	N(alpha)-acetyltransferase 25, NatB auxiliary subunit	231713	-	1.2	-	1	-	-
Nampt	nicotinamide phosphoribosyltransferase	59027	1.7	1.9	1.5	2	-	-
Nlrc5	NLR family, CARD domain containing 5	434341	-	1.8	-	-	-	-
Nmi	N-myc (and STAT) interactor	64685	1.3	1.6	1.1	1.5	-	-
Nod1	nucleotide-binding oligomerization domain containing 1	107607	-	-	-	1	-	-
Nol8	nucleolar protein 8	70930	-	-	-	1	-	-
Nop56	NOP56 ribonucleoprotein	67134	-	-	-	1.4	-	-
Nos2	nitric oxide synthase 2, inducible	18126	-	-	-	1.1	-	-
Oas1a	2'-5' oligoadenylate synthetase 1A	246730	1.5	2.7	1.6	1.4	-	-
Oas1b	2'-5' oligoadenylate synthetase 1B	23961	-	1.6	-	-	-	-
Oas1g	2'-5' oligoadenylate synthetase 1G	23960	2.3	3.9	2.5	2.3	-	-
Oas2	2'-5' oligoadenylate synthetase 2	246728	2.1	3.4	2	1.6	-	-
Oas3	2'-5' oligoadenylate synthetase 3	246727	1.1	2.6	1.3	1	-	-
Oasl1	2'-5' oligoadenylate synthetase-like 1	231655	2.6	3.5	2.4	2.5	-	-
Oasl2	2'-5' oligoadenylate synthetase-like 2	23962	2	3.4	2	2	-	-
Ogfr	opioid growth factor receptor	72075	-	1.4	-	-	-	-
Olfr56	olfactory receptor 56	18356	1.7	2.1	1.2	1.7	-	-
P2ry14	purinergic receptor P2Y, G-protein coupled, 14	140795	-	-	1.3	1.1	-	-
Parp10	poly (ADP-ribose) polymerase family, member 10	671535	1.2	1.5	-	1.2	-	-
Parp11	poly (ADP-ribose) polymerase family, member 11	101187	1	1.4	-	-	-	-
Parp12	poly (ADP-ribose) polymerase family, member 12	243771	1.5	2.5	1.3	1.6	-	-
Parp14	poly (ADP-ribose) polymerase family, member 14	547253	1.1	1.7	-	1	-	-
Parp9	poly (ADP-ribose) polymerase family, member 9	80285	1.3	1.9	1.1	1.2	-	-
Pdcd5	programmed cell death 5	56330	-	-	-	1.2	-	-
Pde7b	phosphodiesterase 7B	29863	1.8	2.3	2	1.6	-	-
Pdk4	pyruvate dehydrogenase kinase, isoenzyme 4	27273	1.5	1.1	1.7	1.4	1.8	1.8
Pf4	platelet factor 4	56744	-	-	1.2	1.1	-	-
Pfkp	phosphofructokinase, platelet	56421	-	1	-	1.1	-	-
Phf11a	PHD finger protein 11A	219131	1.2	1.4	1.1	1.1	-	-

Phf11b	PHD finger protein 11B	236451	2	1.8	1.7	1.8	-	-
Phf11c	PHD finger protein 11C	628705	2	2.4	1.7	1.7	-	-
Phf11d	PHD finger protein 11D	219132	2.4	2.9	2.4	2.4	1.1	-
Pi4kb	phosphatidylinositol 4-kinase, catalytic, beta polypeptide	107650	1.4	1.4	1.3	1.2	-	-
Pkib	protein kinase inhibitor beta, cAMP dependent, testis specific	18768	1.2	1.4	-	1.2	-	-
Pla2g4a	phospholipase A2, group IVA (cytosolic, calcium-dependent)	18783	-	-	-	1.2	-	-
Plac8	placenta-specific 8	231507	1.3	1.7	-	1.3	-	-
Plau	plasminogen activator, urokinase	18792	1.2	1.1	-	1.1	-	-
Plin2	perilipin 2	11520	1.3	1.5	1.4	1.5	1.1	-
Pno1	partner of NOB1 homolog (S. cerevisiae)	66249	-	1.1	-	1.1	-	-
Pnp	purine-nucleoside phosphorylase	18950	-	1.2	1.2	1.3	-	-
Pnpt1	polyribonucleotide nucleotidyltransferase 1	71701	1.1	-	1.1	1.4	-	-
Pomp	proteasome maturation protein	66537	-	-	1	-	-	-
Pop4	processing of precursor 4, ribonuclease P/MRP family, (S. cerevisiae)	66161	-	-	1	1.2	-	-
Ppa1	pyrophosphatase (inorganic) 1	67895	1.3	2.1	-	1.5	-	-
Pram1	PML-RAR alpha-regulated adaptor molecule 1	378460	-	-	1.1	1.1	-	-
Prdx1	peroxiredoxin 1	18477	1	-	1	1.2	-	-
Prf1	perforin 1 (pore forming protein)	18646	1.2	1.4	1.1	1.2	-	-
Prmt1	protein arginine N-methyltransferase 1	15469	-	-	-	1	-	-
Procr	protein C receptor, endothelial	19124	1	-	-	1	-	-
Psat1	phosphoserine aminotransferase 1	107272	1.3	1.4	1	1.3	-	-
Psma1	proteasome (prosome, macropain) subunit, alpha type 1	26440	-	-	1.1	1.3	-	-
Psma7	proteasome (prosome, macropain) subunit, alpha type 7	26444	-	1.1	-	1.1	-	-
Psmb10	proteasome (prosome, macropain) subunit, beta type 10	19171	1.1	1.4	-	1.3	-	-
Psmb8	proteasome (prosome, macropain) subunit, beta type 8 (large multifunctional peptidase 7)	16913	-	1.4	-	1.1	-	-
Psmc6	proteasome (prosome, macropain) 26S subunit, ATPase, 6	67089	-	-	-	1	-	-
Psme1	proteasome (prosome, macropain) 28 subunit, alpha	19186	-	1.1	-	1	-	-
Ptgs2	prostaglandin-endoperoxide synthase 2	19225	2.3	2	1.4	2	1.9	-
Pydc3	pyrin domain containing 3	100033459	1.9	2.4	1.7	1.5	-	-
Pydc4	pyrin domain containing 4	623121	2.9	3.4	2.2	1.9	-	-
Pyhin1	pyrin and HIN domain family, member 1	236312	1.7	1.8	1.5	1.4	-	-
Rasa4	RAS p21 protein activator 4	54153	-	1.1	1	1	-	-
Retnlg	resistin like gamma	245195	1.1	1.3	1.5	1.6	1.4	1.6
Rgs1	regulator of G-protein signaling 1	50778	1.7	1.7	1.4	1.5	1.2	-
Rnf19b	ring finger protein 19B	75234	-	1.2	-	1.1	-	-
Rnf213	ring finger protein 213	672511	1.4	2.2	1	1	-	-
Rpf2	ribosome production factor 2 homolog (S. cerevisiae)	67239	-	1.2	-	1.3	-	-
Rpl23a	ribosomal protein L23A	268449	-	-	-	1.3	-	-
Rsl1d1	ribosomal L1 domain containing 1	66409	-	-	-	1.2	-	-
Rtp4	receptor transporter protein 4	67775	1.4	2.2	1.2	1.1	-	-
Rundc3b	RUN domain containing 3B	242819	-	1	-	-	-	-
S100a8	S100 calcium binding protein A8 (calgranulin A)	20201	-	1.4	1.6	1.9	-	-
S100a9	S100 calcium binding protein A9 (calgranulin B)	20202	-	1.2	-	1.2	-	-
Scarna6	small Cajal body-specific RNA 6	1.00E+08	-	-	-1.6	-	-	-
Scimp	SLP adaptor and CSK interacting membrane protein	327957	1.2	-	1.2	1.5	-	-
Sco1	SCO cytochrome oxidase deficient homolog 1 (yeast)	52892	1.5	1.6	1.4	1.6	-	-
Sepw1	selenoprotein W, muscle 1	20364	1.1	1.2	1.1	1.1	-	-
Serpina3f	serine (or cysteine) peptidase inhibitor, clade A, member 3F	238393	1.9	2.7	1.3	2.2	1.3	-
Serpinb6b	serine (or cysteine) peptidase inhibitor, clade B, member 6b	20708	-	-	-	1.2	-	-
Serpinb9	serine (or cysteine) peptidase inhibitor, clade B, member 9	20723	1.3	1.1	1.1	1.4	-	-
Serpinb9b	serine (or cysteine) peptidase inhibitor, clade B, member 9b	20706	1.2	1	1.1	-	-	-
Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1	18787	1.1	-	-	1	-	-
Setdb2	SET domain, bifurcated 2	239122	1.3	1.8	1.1	1.1	-	-
Sfrp1	secreted frizzled-related protein 1	20377	1	1	1.1	1.2	-	-
Sgcb	sarcoglycan, beta (dystrophin-associated	24051	1.7	1.5	1.8	2	-	-

	glycoprotein)							
Slamf8	SLAM family member 8	74748	-	1.1	-	1.2	-	-
Slc15a3	solute carrier family 15, member 3	65221	-	1.3	-	1.1	-	-
Slc25a22	solute carrier family 25 (mitochondrial carrier, glutamate), member 22	68267	-	1.2	1	1.2	-	-
Slfn1	schlafen 1	20555	1.8	1.9	1.3	1.5	-	-
Slfn2	schlafen 2	20556	-	1.1	-	-	-	-
Slfn3	schlafen 3	20557	1.3	1.7	1.5	1.7	-	-
Slfn4	schlafen 4	20558	1.9	3	2	1.8	-	-
Slfn5	schlafen 5	327978	1.5	2.4	1.4	1.1	-	-
Slfn8	schlafen 8	276950	1.2	1.5	-	-	-	-
Slfn9	schlafen 9	237886	1.5	2.4	1.4	1.7	-	-
Snora81	small nucleolar RNA, H/ACA box 81	1.00E+08	-	1.1	-	-	-	-
Snord12	small nucleolar RNA, C/D box 12	1.00E+08	-	1.9	-	-	-	-
Snord52	small nucleolar RNA, C/D box 52	1.00E+08	-	1.2	-	-	-	-
Snord72	small nucleolar RNA, C/D box 72	1.00E+08	-	1.4	-	-	-	-
Snrpb2	U2 small nuclear ribonucleoprotein B	20639	-	-	1.1	1	-	-
Snx10	sorting nexin 10	71982	-	1.2	-	1.1	-	-
Snx6	sorting nexin 6	72183	-	-	1.1	1.3	-	-
Socs1	suppressor of cytokine signaling 1	12703	1.6	2.2	-	1.9	-	-
Socs2	suppressor of cytokine signaling 2	216233	1	1.4	-	1.8	-	-
Sp100	nuclear antigen Sp100	20684	1.1	1.7	1.4	-	-	-
Sp100	nuclear antigen Sp100	20684	-	1.4	-	-	-	-
Sp140	Sp140 nuclear body protein	434484	-	1.1	-	-	-	-
Spon1	spondin 1, (f-spondin) extracellular matrix protein	233744	1.6	1.9	1.5	1.7	-	-
Stat1	signal transducer and activator of transcription 1	20846	-	1.4	-	-	-	-
Stat2	signal transducer and activator of transcription 2	20847	1.4	1.9	1.2	1.5	-	-
Stxbp3a	syntaxin binding protein 3A	20912	1.1	1.1	1.3	1.2	-	-
Taf1d	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, D	75316	-	2.4	-	-	-	-
Tap1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	21354	-	1.1	-	-	-	-
Tap2	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	21355	-	1	-	-	-	-
Tdrd7	tudor domain containing 7	100121	-	1.3	-	-	-	-
Tfec	transcription factor EC	21426	1.6	1	1.7	1.6	-	-
Tgm2	transglutaminase 2, C polypeptide	21817	1.3	1.7	1.1	1.5	1	-
Tgtp1	T cell specific GTPase 1	21822	-	1.4	-	1.1	-	-
Tgtp2	T cell specific GTPase 2	1.00E+08	1.6	2.7	-	1.5	-	-
Timp1	tissue inhibitor of metalloproteinase 1	21857	2.8	2.9	2.4	2.9	1.8	1.6
Tlr13	toll-like receptor 13	279572	1.4	1	1.7	1.5	1	-
Tlr3	toll-like receptor 3	142980	1	1.4	1.2	1.1	-	-
Tlr7	toll-like receptor 7	170743	1.1	1.2	1.2	-	-	-
Tlr8	toll-like receptor 8	170744	1.1	1.1	1.3	1	-	-
Tm4sf1	transmembrane 4 superfamily member 1	17112	-	-	-	1	-	-
Tmem106a	transmembrane protein 106A	217203	-	1.1	-	1.1	-	-
Tmem184b	transmembrane protein 184b	223693	-	1.1	-	-	-	-
Tmem67	transmembrane protein 67	329795	1.3	1.4	1.2	1.4	-	-
Tnfsf10	tumor necrosis factor (ligand) superfamily, member 10	22035	2	2.3	2	1.9	-	-
Tnn	tenascin N	329278	-	1.2	-	-	-	-
Tomm70a	translocase of outer mitochondrial membrane 70 homolog A (yeast)	28185	-	1.1	-	-	-	-
Tor3a	torsin family 3, member A	30935	1.6	2.1	1.4	1.4	-	-
Trafd1	TRAF type zinc finger domain containing 1	231712	1.1	1.6	1	1.1	-	-
Trem3	triggering receptor expressed on myeloid cells 3	58218	1.1	1.4	1.2	1.5	-	-
Trex1	three prime repair exonuclease 1	22040	-	1.2	-	1	-	-
Trim12c	tripartite motif-containing 12C	319236	-	1.1	-	-	-	-
Trim12c	tripartite motif-containing 12C	319236	-	1.5	1.2	1.3	-	-
Trim21	tripartite motif-containing 21	20821	1.1	1.3	-	1.4	-	-
Trim25	tripartite motif-containing 25	217069	-	1	-	-	-	-
Trim30a	tripartite motif-containing 30A	20128	1.3	2.1	-	1.1	-	-
Trim30c	tripartite motif-containing 30C	434219	2.6	3.4	2.5	2.2	-	-
Trim30d	tripartite motif-containing 30D	209387	3.1	3.5	3.4	2.7	1.4	-
Trim34b	tripartite motif-containing 34B	434218	-	1.1	-	-	-	-
Tspo	translocator protein	12257	1.1	1.4	1.3	1.3	-	-
Tubb1	tubulin, beta 1 class VI	545486	-	-	-	1.3	-	-
Uba7	ubiquitin-like modifier activating enzyme 7	74153	-	1.1	-	-	-	-
Upp1	uridine phosphorylase 1	22271	-	1.1	-	-	-	-
Usp18	ubiquitin specific peptidase 18	24110	2.2	3.1	2	1.8	-	-

Vwa5a	von Willebrand factor A domain containing 5A	67776	-	1.3	-	1.2	-	-
Wars	tryptophanyl-tRNA synthetase	22375	1.1	1.6	-	1.4	-	-
Wfdc17	WAP four-disulfide core domain 17	1.00E+08	2.7	2.4	2.8	2.7	-	-
Xaf1	XIAP associated factor 1	327959	1.3	2.1	1.1	1.2	-	-
Xdh	xanthine dehydrogenase	22436	2.3	3	2.4	2.4	1.2	-
Zbp1	Z-DNA binding protein 1	58203	1.9	2.7	1.3	1.6	-	-
Zcchc2	zinc finger, CCHC domain containing 2	227449	-	1.1	-	-	-	-
Zfp1	zinc finger protein 1	22640	-	1.1	1.1	1.2	-	-
Znfx1	zinc finger, NFX1-type containing 1	98999	1	1.6	1	1.1	-	-
Znrd1	zinc ribbon domain containing, 1	66136	-	-	1.1	1.2	-	-
Zufsp	zinc finger with UFM1-specific peptidase domain	72580	-	1.5	-	-	-	-

Table a2. Full list of annotated down regulated genes in mouse spleens in response to canarypox virus (CNPV), pigeonpox (FeP2), fowlpox virus (FWPV), lumpy skin disease virus (LSDV), modified vaccinia Ankara (MVA) and penguinpox virus (PEPV). Differences in Log_2 Fold Changes (between each virus and the control) are depicted.

Symbol	Name	Entrez	MVA	LSDV	CNPV	FWPV	PEPV	FeP2
2510003D18Rik	RIKEN cDNA 2510003D18 gene	72317	-1.1	-1.3	-	-	-	-
2900052N01Rik	RIKEN cDNA 2900052N01 gene	73040	-	-1.1	-	-	-	-
5430401H09Rik	RIKEN cDNA 5430401H09 gene	100504461	-1.5	-1.1	-	-	-	-
9430076G02Rik	RIKEN cDNA 9430076G02 gene	77433	-2.2	-1.8	-1.9	-1.9	-1.5	-1.3
A530099J19Rik	RIKEN cDNA A530099J19 gene	319293	-1.3	-1.5	-	-	-	-
Abca9	ATP-binding cassette, sub-family A (ABC1), member 9	217262	-1.3	-1.5	-	-1.1	-	-
Adam23	a disintegrin and metallopeptidase domain 23	23792	-1.3	-1.3	-1.0	-1.2	-	-
Adamdec1	ADAM-like, decysin 1	58860	-	-1.1	-	-	-	-
Add2	adducin 2 (beta)	11519	-1.3	-	-1.1	-	-	-
Ano1	anoctamin 1, calcium activated chloride channel	101772	-1.4	-1.4	-	-	-	-
Aplnr	apelin receptor	23796	-1.4	-1.5	-1.2	-1.2	-	-
Aqp1	aquaporin 1	11826	-1.5	-1.2	-1.2	-1.1	-	-
Aspm	asp (abnormal spindle)-like, microcephaly associated (Drosophila)	12316	-1.2	-	-	-	-	-
Atp2b4	ATPase, Ca++ transporting, plasma membrane 4	381290	-1.4	-	-1.1	-1.1	-	-
B3gnt8	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 8	232984	-1.3	-1.3	-1.1	-1.1	-	-
Btnl10	butyrophilin-like 10	192194	-1.8	-1.3	-1.2	-1.2	-1.1	-
Cacna1g	calcium channel, voltage-dependent, T type, alpha 1G subunit	12291	-1.3	-	-	-	-	-
Ccdc80	coiled-coil domain containing 80	67896	-1.4	-1.4	-1.3	-	-1.1	-
Ccl21a	chemokine (C-C motif) ligand 21A (serine)	18829	-1.3	-1.7	-	-	-	-
Ccl21a	chemokine (C-C motif) ligand 21A (serine)	18829	-1.3	-1.7	-	-	-	-
Ccl21b	chemokine (C-C motif) ligand 21B (leucine)	100042493	-1.2	-1.6	-	-	-	-
Ccl21b	chemokine (C-C motif) ligand 21B (leucine)	100042493	-1.3	-1.6	-	-	-	-
Ccl21b	chemokine (C-C motif) ligand 21B (leucine)	100042493	-1.2	-1.7	-	-	-	-
Ccl21b	chemokine (C-C motif) ligand 21B (leucine)	100042493	-1.2	-1.7	-	-	-	-
Ccl21b	chemokine (C-C motif) ligand 21B (leucine)	100042493	-1.2	-1.7	-	-	-	-
Ccl21b	chemokine (C-C motif) ligand 21B (leucine)	100042493	-1.2	-1.7	-	-	-	-
Ccl21c	chemokine (C-C motif) ligand 21C (leucine)	65956	-1.2	-1.6	-	-	-	-
Cd209a	CD209a antigen	170786	-1.7	-2.2	-1.1	-1.5	-	-
Cd209b	CD209b antigen	69165	-1.2	-1.3	-	-	-	-
Cd59a	CD59a antigen	12509	-	-1.2	-	-	-	-
Cd7	CD7 antigen	12516	-	-1.1	-	-	-	-
Cdh8	cadherin 8	12564	-	-1.0	-	-	-	-
Cdkl1	cyclin-dependent kinase-like 1 (CDC2-related kinase)	71091	-1.2	-	-	-	-	-
Cdkn2c	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	12580	-1.3	-	-	-	-	-
Cep76	centrosomal protein 76	225659	-1.1	-	-	-	-	-
Cit	citron	12704	-1.3	-	-	-	-	-
Cldn13	claudin 13	57255	-1.5	-1.2	-1.1	-	-	-
Col14a1	collagen, type XIV, alpha 1	12818	-1.6	-1.6	-1.3	-1.4	-1.0	-
Cpm	carboxypeptidase M	70574	-1.3	-1.3	-1.1	-1.1	-	-
Csmd3	CUB and Sushi multiple domains 3	239420	-1.0	-1.1	-	-	-	-
Ctsf	cathepsin F	56464	-1.1	-	-	-	-	-
Depdc1a	DEP domain containing 1a	76131	-1.1	-	-	-	-	-
Diap3	diaphanous homolog 3 (Drosophila)	56419	-1.1	-	-	-	-	-
E2f2	E2F transcription factor 2	242705	-1.5	-	-1.2	-1.2	-	-
E2f8	E2F transcription factor 8	108961	-1.4	-1.0	-1.1	-	-	-
Emr4	EGF-like module containing, mucin-like, hormone receptor-like sequence 4	52614	-1.4	-1.8	-1.1	-1.5	-1.3	-
Esm1	endothelial cell-specific molecule 1	71690	-	-1.1	-	-	-	-
Fbln5	fibulin 5	23876	-2.0	-2.0	-1.4	-1.3	-1.1	-
Fcamr	Fc receptor, IgA, IgM, high affinity	64435	-1.1	-	-1.5	-	-	-
Fcer2a	Fc receptor, IgE, low affinity II, alpha polypeptide	14128	-2.6	-3.1	-2.4	-2.5	-2.2	-1.3
Fhdc1	FH2 domain containing 1	229474	- 1.42824	-	-	-	-	-
Fn3k	fructosamine 3 kinase	63828	-2.0	-1.5	-1.5	-1.5	-1.1	-
Gas6	growth arrest specific 6	14456	-	-1.1	-	-	-	-
Glrx5	glutaredoxin 5 homolog (S. cerevisiae)	73046	-1.3	-	-1.1	-	-	-
Gm12839	cytochrome P450, family 4, subfamily b, polypeptide 1 pseudogene	631037	-1.7	-1.3	-1.1	-1.4	-1.4	-
Gm20236	predicted gene, 20236	100504453	-1.9	-1.2	-1.5	-1.6	-1.3	-
Gm20236	predicted gene, 20236	100504453	-1.9	-1.2	-1.5	-1.6	-1.3	-
Gpsm2	G-protein signalling modulator 2 (AGS3-like, C. elegans)	76123	-1.1	-	-	-	-	-
H2-M2	histocompatibility 2, M region locus 2	14990	-1.1	-1.4	-1.0	-1.0	-1.1	-
Hmmr	hyaluronan mediated motility receptor (RHAMM)	15366	-1.0	-	-	-	-	-

Hs3st2	heparan sulfate (glucosamine) 3-O-sulfotransferase 2	195646	-1.4	-1.3	-1.0	-1.0	-	-
Ifi27l1	interferon, alpha-inducible protein 27 like 1	52668	-1.4	-1.3	-	-	-	-
Igfbp3	insulin-like growth factor binding protein 3	16009	-	-1.0	-	-	-	-
Igk	immunoglobulin kappa chain complex	243469	-	-1.0	-	-	-	-
Kcnj16	potassium inwardly-rectifying channel, subfamily J, member 16	16517	-	-1.1	-	-	-	-
Kel	Kell blood group	23925	-1.5	-1.5	-	-	-	-
Kif14	kinesin family member 14	381293	-1.3	-	-	-	-	-
Kif23	kinesin family member 23	71819	-1.1	-	-	-	-	-
Klhl14	kelch-like 14	225266	-1.5	-1.6	-1.3	-1.4	-1.2	-
Kynu	kynureninase (L-kynurenine hydrolase)	70789	-1.3	-1.4	-1.0	-1.1	-	-
Lilra5	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 5	232801	-	-1.1	-	-	-	-
Limch1	LIM and calponin homology domains 1	77569	-1.1	-1.2	-	-	-	-
Lphn3	latrophilin 3	319387	-	-1.1	-	-	-	-
Mgll	monoglyceride lipase	23945	-1.3	-	-	-	-	-
Mgst3	microsomal glutathione S-transferase 3	66447	-1.6	-1.0	-	-1.0	-	-
Mir687	microRNA 687	751541	-1.4	-	-	-	-	-
Nusap1	nucleolar and spindle associated protein 1	108907	-1.1	-	-	-	-	-
Nxpe4	neurexophilin and PC-esterase domain family, member 4	244853	-1.2	-	-	-	-	-
Olfml2a	olfactomedin-like 2A	241327	-	-1.0	-	-	-	-
Paqr9	progestin and adipoQ receptor family member IX	75552	-1.1	-	-	-	-	-
Pcp4	Purkinje cell protein 4	18546	-1.2	-1.5	-	-	-	-
Pcx	pyruvate carboxylase	18563	-1.4	-	-	-	-	-
Pkhd1l1	polycystic kidney and hepatic disease 1-like 1	192190	-2.6	-2.0	-2.1	-2.1	-1.7	-
Pklr	pyruvate kinase liver and red blood cell	18770	-1.1	-	-	-	-	-
Ppox	protoporphyrinogen oxidase	19044	-1.1	-1.1	-	-	-	-
Prelp	proline arginine-rich end leucine-rich repeat	116847	-1.0	-1.2	-	-	-	-
Prkcg	protein kinase C, gamma	18752	-	- 1.02954	-	-	-	-
Reep6	receptor accessory protein 6	70335	-1.1	-	-	-	-	-
Sec14l2	SEC14-like 2 (S. cerevisiae)	67815	-1.7	-1.3	-1.5	-1.3	-1.2	-
Sh3yl1	Sh3 domain YSC-like 1	24057	-1.6	-1.2	-	-	-	-
Slc12a2	solute carrier family 12, member 2	20496	-1.0	-1.0	-	-	-	-
Slc16a10	solute carrier family 16 (monocarboxylic acid transporters), member 10	72472	-1.4	-	-	-1.1	-	-
Slc2a4	solute carrier family 2 (facilitated glucose transporter), member 4	20528	-1.7	-1.0	-	-1.1	-	-
Slc38a5	solute carrier family 38, member 5	209837	-1.4	-1.2	-1.0	-	-	-
Slc6a20a	solute carrier family 6 (neurotransmitter transporter), member 20A	102680	-1.4	-1.3	-1.0	-1.0	-	-
Sned1	sushi, nidogen and EGF-like domains 1	208777	-1.1	-1.1	0	-1.0	-	-
Snx22	sorting nexin 22	382083	-1.4	-1.1	-	-	-	-
Sox6	SRY-box containing gene 6	20679	-1.8	-1.2	-1.2	-1.4	-1.1	-
Sptb	spectrin beta, erythrocytic	20741	-1.9	-1.4	-1.6	-1.5	-1.3	-1.3
St8sia6	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 6	241230	-	-1.0	-	-	-	-
Tac2	tachykinin 2	21334	-1.0	-1.5	-	-	-	-
Tfrc	transferrin receptor	22042	-1.2	-	-	-	-	-
Timd4	T cell immunoglobulin and mucin domain containing 4	276891	-1.1	-1.3	-	-	-	-
Tlr11	toll-like receptor 11	239081	-1.0	-	-	-	-	-
Tspan33	tetraspanin 33	232670	-1.6	-	-1.3	-1.1	-	-
Tspan8	tetraspanin 8	216350	-1.4	-	-	-1.0	-	-

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